

EXHIBIT A

Docket No.: 023004.0103X1US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Reexamination Application of:
Michael W. Graham et al.

Application No.: 90/007247

Confirmation No.: 6310

Filed: October 4, 2004

Art Unit: 1639

For: GENETIC CONSTRUCTS FOR DELAYING
OR REPRESSING THE EXPRESSION OF A
TARGET GENE

Examiner: B. M. Celsa

DECLARATION UNDER 37 C.F.R. § 1.131

Customer Window, MS Amendment
U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Dear Sir:

I, Michael Graham, Ph.D., declare as follows:

1. I am a named inventor of the subject matter in the above-identified reexamination. I am a resident and citizen of Australia. During the period of December 1997 through the filing of the priority document for the patent under reexamination, I was a research scientist in Australia. During this period Robert Rice and Margaret Bernard were under my direction and supervision.

2. I have reviewed the above-identified reexamination, including the present claims. As I understand it, the presently claimed subject matter is generally directed to genetic constructs that are capable of delaying, repressing or otherwise reducing the expression of a target gene in an animal cell, as well as methods for using these constructs and animal cells comprising these constructs. I understand that the presently claimed constructs comprise at least one structural gene sequence placed operably in a sense orientation under the control of a

promoter and at least one structural gene sequence placed operably in an antisense orientation under the control of a promoter, where the structural gene sequences comprise a nucleotide sequence which is substantially identical to at least a region of a target gene, and where

- a. the multiple structural gene sequences are placed operably under the control of a single promoter sequence, where optionally the structural gene sequences in sense and antisense orientations are spaced from each other by a nucleic acid stuffer fragment; or
- b. the structural gene sequences in sense and antisense orientations are each placed operably under the control of individual promoter sequences.

3. I am aware of the rejections issued in an Office Action mailed January 24, 2007, in the pending reexamination. I understand that a rejection in the Office Action was based on the teachings of Fire U.S. Patent No. 6,506,559 (the "Fire reference"). The Fire reference was filed in late 1998, and claims priority to U.S. Serial No. 60/068,562 (the "Fire priority application") filed December 23, 1997, less than a year before the effective filing date of the patent under reexamination in the United States. It is my understanding that to show prior invention, the Examiner is requiring that I provide evidence of conception prior to the date of filing of the Fire priority application and then the Examiner is requiring that I show diligence from just before the filing date until reduction to practice or constructive reduction to practice of my own invention.

4. Exhibit I is a copy of laboratory notebook pages showing my preliminary work in plants. My early work on genetic constructs for reducing expression of a target gene was in plants and I spent significant amounts of time trying to produce such constructs. I consider this work important to my present invention because the layout of the constructs in plants was the basis for my later attempt in animals. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. I conceived the subject matter of the presently claimed invention prior to December 23, 1997. Evidence for conception before the date of the Fire priority application includes laboratory notebook pages 107 – 108, which show one

of my first attempts to make a genetic construct which was designed to express both sense and antisense RNAs from multiple copies of a nucleotide sequence under the control of a promoter. Page 108 in particular shows my drawing of a "Hairpin GUS" construct that includes two copies of a structural gene sequence in the antisense and sense orientation, expression of which was driven by a single promoter. Laboratory notebook pages 110 – 121 show my additional experiments to build constructs with structural gene sequences in a sense and antisense orientation. Laboratory notebook pages 130 – 32 show experiments where I attempted to make expression cassettes containing two promoters designed to express separate sense and antisense RNAs. Laboratory notebook pages 138, 145, 147, 150, 151, 159, 169, 175, 181-192, 206, 216, 229 and 266 show experiments where I continued to try and create genetic constructs expressing separate sense and antisense RNAs. Laboratory notebook pages 138, 145, 150, 153, 158, 165, 168, 172, 175, 182, 185, 195, 197, 198, 200, 210, 227, 229, 240 and 254 show experiments where I developed constructs in which the structural genes were orientated in a sense and antisense orientation, some of which were controlled by separate promoters. I understand that this is evidence of a conception of genetic constructs of the same type as those of the claimed invention earlier than the priority date of Fire rather than the earliest conception of the claimed invention, which occurred before these notebook entries.

5. Further evidence of conception before the date of the Fire priority application includes the June 6, 1994 letter from CSIRO to John Slattery, as indicated in Exhibit 2. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. In this letter, my employer at the time, CSIRO, requested from Mr. Slattery an opinion on the patentability of my new constructs that I believed would "be useful in mammalian systems", as I indicated on the last page of the attachment. Attached to the letter is my idea to create constructs such as the subject matter in the above-identified reexamination. For instance, the second figure, Case 2, is a construct comprising a single promoter that transcribes two structural gene sequences in an inverted repeat to form a transcript with a "hairpin" structure, where the inverted repeat is not separated by a stuffer fragment. This figure shows my idea of making a construct like the construct of Claim 3. The third figure, Case 3, is a construct comprising two copies of a structural gene sequence, where one copy is placed operably in the sense

orientation under the control of an individual promoter and the other copy is placed operably in the antisense orientation under the control of a separate promoter. Case 3 therefore shows my idea of making the construct of Claim 4 and indicates I had conceived of this invention on or before June 6, 1994.

6. Exhibit 3 is an early outline for a provisional patent application which I prepared on August 8, 1995. In this draft, I discuss decreasing gene expression in animals by use of novel transgene designs. This Exhibit was previously submitted in the June 12, 2006 37 C.F.R. § 1.131 declaration.
7. Exhibit 4 is a draft of an unpublished manuscript which I prepared on June 21, 1996. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. I prepared this article for publication in a journal to try and demonstrate the widespread existence of an RNA degradative system in plants and other organisms based on a re-interpretation of the literature that existed at that time. While genetic constructs were not described in this document, their development was based on ideas presented therein, specifically I wished to design constructs to switch this RNA degradative system on more efficiently in transgenic organisms. I discussed evidence that this RNA degradative system existed in animals in the section titled "Post-transcriptional gene inactivation in other taxa" and believe this document demonstrates my thinking at that time, namely the types of constructs that might work in plants would also work in animals.
8. Exhibit 5 is a draft of a proposal I prepared on November 29, 1996, proposing that genetic constructs for gene silencing would prove effective in animals. This proposal was important because budgetary limits at Benitec (then called Ag-Genes and my employer at that time) inhibited my ability to conduct all of the research I intended for target gene inactivation. In this proposal, I discuss the genetic constructs I previously created for plants and how I wanted to create "multiple gene constructs, the use of direct and inverted sequences and the design and use of RNA stabilizing sequences" to decrease gene expression in animals. When our funding increased, we promptly hired Robert Rice to work on post-transcriptional gene silencing in animal cells.

9. Exhibit 6 shows further evidence of my conception before the date of the Fire priority application. This Exhibit was previously submitted in the June 12, 2006, 37 C.F.R. § 1.131 declaration. Laboratory notebook pages 52 – 55, which are from May 1997, show how I conceived a genetic construct design to express hairpin RNAs where the construct had a stuffer fragment inserted between the two copies of the nucleotide sequence, where one of the sequences was in a sense orientation and the other sequence was in an antisense orientation relative to the promoter. I consider this Exhibit important because previously I had difficulty creating inverted repeat constructs without a stuffer fragment due to instability of such constructs in *E. coli*. The insertion of the stuffer fragment between the inverted repeat sequences allowed me to readily make such constructs, and as such I planned to incorporate this idea into the genetic constructs for reducing expression of animal genes. Thus, compared to constructs without a stuffer fragment, the inverted repeat constructs with a stuffer fragment were superior.

10. I understand the Examiner would like to see the diligence to reduce my invention to practice between the Fire priority date of December 23, 1997, and the '099 patent priority date of March 20, 1998. As such, I detail below the events that occurred. To assist the Examiner, I also include a calendar of December 1997, and January – March 1998.

December 1997

Su	Mo	Tu	We	Th	Fr	Sa
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28	29	30	31			
7:00	13:00	21:00	29:00			

January 1998							February 1998							March 1998						
Su	Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa
				1	2	3	1	2	3	4	5	6	7	1	2	3	4	5	6	7
4	5	6	7	8	9	10	8	9	10	11	12	13	14	8	9	10	11	12	13	14
11	12	13	14	15	16	17	15	16	17	18	19	20	21	15	16	17	18	19	20	21
18	19	20	21	22	23	24	22	23	24	25	26	27	28	22	23	24	25	26	27	28
25	26	27	28	29	30	31								29	30	31				
5:0	12:0	20:0	28:0				3:0	11:0	19:0	26:0				5:0	12:0	21:0	27:0			

11. When Ag-Gene funding increased in late 1997, we promptly pursued hiring Robert Rice to work on preparing gene constructs for gene silencing in animal cells, corresponding to the designs I had conceived. From what I recall, we at Ag-Gene started discussing hiring Dr. Rice in October 1997. We wanted to work with Dr. Rice because he had extensive experience in a range of molecular biological techniques and plasmid design and construction. Dr. Rice's thesis topic was eukaryotic evolution and studying eukaryotic divergence using ribosomal RNA sequence data and secondary structure remodeling. As such, Dr. Rice also had experience with use of computers for systematic / bioinformatics analysis of DNA / RNA sequences.

12. On December 8, 1997, I decided to target the polymerase gene of the bovine enterovirus (BEV) as an exemplary target gene in animal cells. This gene was chosen because it could be easily determined whether the expression of constructs based on the gene had an effect on viral replication in animal cells. Specifically, since infection of Mabin Darby (MDBK) cells with BEV normally kills them, we could therefore determine whether expression of constructs in transformed cells might inhibit viral replication simply by determining whether such transformed cells show prolonged survival following challenge with the virus under standardised conditions. Further, we knew that the BEV polymerase may be amplified using the polymerase chain reaction or alternatively, isolated using standard hybridisation techniques. With the assistance of Margaret Bernard ("Ms. Bernard"), I printed out the sequence of the polymerase gene of BEV, see Exhibit 7, page 2. (indicating the sequence was

printed at 3:13pm on December 8, 1997). Again with the assistance of Ms. Bernard, I designed a pair of oligonucleotide primers to amplify a region of the BEV gene. These primers, designated BEV-1 and BEV-2 (pages 2-3 of Exhibit 7) were ordered by Ms. Bernard from a commercial supplier under my instruction on December 9, 1997. (*See, Id.* at 1, lower entry: the notation the primers were ordered December 9, 1998 is in error; they were ordered December 9, 1997 as evidenced by their entry on the notebook page of December 9, 1997 and their use on January 6, 1998). These primers were available for use by Ms. Bernard on January 6, 1998. We continued with BEV as a target gene all the way to actually practicing the invention, as can be seen in the figures in the patent application that we filed.

13. On or about December 8, 1997, I mentioned to Ms. Bernard that as soon as possible she would be devoting a greater amount of her time for work on a project with the new Research Scientist, Dr. Rice, in preparing the gene constructs for the animal target gene, in particular the constructs targeting BEV.

14. Dr. Rice arrived to commence employment on the "animal project" on December 21, 1997. On that day or the day after, I met with Dr. Rice and described to him in detail the types of constructs that I had envisaged for reducing expression of a target gene. The first type of construct was an inverted palindrome construct without a stuffer fragment. Claim 3 of my patent under this reexamination is to the inverted palindrome construct without the stuffer fragment and claim 7 is to a method of using the construct. The second type of construct was an inverted palindrome construct with a stuffer fragment. Claim 5 is to the inverted palindrome construct with the stuffer fragment and claim 9 is to a method of using the construct. Finally, I wanted to make a construct with two copies of a gene sequence where each copy was under the control of a separate promoter. Claim 4 is to this type of construct and claim 10 is to a method of using this construct. On the days following, I had further discussions with Dr. Rice about these types of constructs.

15. The laboratory facilities of Ag-Gene were located at the Queensland Agricultural Biotechnology Centre (QABC), an operational centre of the Queensland State Government's Department of Primary Industries. The Queensland State Government provided paid leave

for Christmas day (December 25), Boxing Day (December 26) and New Year's Day (January 1). Further, the Queensland State Government mandated that all State Government employees do not work on the days between December 26 and January 1. As such, the QABC laboratories and offices were closed from December 25, 1997 to January 1, 1998.

16. Dr. Rice and I met several times between December 21, 1997 and mid-January 1998 to discuss cosuppression in animal cells and the types of DNA constructs we wanted to prepare. We decided to build a range of constructs with the following structures: linear repeats, that is constructs containing a block of repeated DNA sequences in sense or in an antisense orientation; inverted repeats, that is constructs containing two inverted DNA sequences either with or without a DNA spacer sequence inserted between the inverted sequences; and a construct with two promoters expressing a sequence in the sense and antisense format.

17. From January 1998 to March 1998, Dr. Rice designed approximately 40 plasmid constructs. Exhibit 8 contains approximately 35 plasmid constructs he designed, most of which are also found as figures of the '099 patent.

18. When Ms. Bernard returned from her Christmas vacation on January 5 or 6, 1998, Dr. Rice and I informed her that we wanted her to prepare certain BEV constructs. We described the kind of constructs we wanted, namely the three constructs discussed above in paragraph 14. Ms. Bernard, with my assistance, was to start preparing the BEV constructs. *See*, Exhibit 7 at page 1. In the meantime, Dr. Rice was to use a computer program to design further genetic constructs. Dr. Rice and I explained to Ms. Bernard that the overall aims of the experiments were to "use Bovine enterovirus as a model system to study cosuppression in mammalian cells," which Ms. Bernard recorded in her laboratory notebook at page 2. Ms. Bernard took further notes from our talk, writing down the polymerase gene from BEV was to be used as the sequence for the animal constructs. *Id.* Ms. Bernard states in her notes that once the constructs were prepared, she was going to "transfect mammalian cell line with constructs, probably using the Mabin Darby Bovine Kidney (MDBK) endothelial cell line." *Id.* The cells would then be challenged with BEV. *Id.* Ms. Bernard then describes the initial constructs. *Id.*

19. The first construct to be made was a BEV polymerase-GFP gene fusion in the vector pEGFP-N1. *Id.* In this arrangement, the CMV promoter of pEGFP-N1 lay upstream of the BEV sequence, while the EGFP sequence was placed downstream of and joined to the BEV sequence. Both the BEV and EGFP sequences were designed to be transcribed conjointly by the CMV promoter. The GFP domain was to be used as a marker to indicate BEV-pol positive cells lines and determine whether cosuppression could be detected by transient transfection of BEV-pol positive cells with GFP cDNA. The next construct was similar to the BEV polymerase-GFP fusion construct above, except that the EGFP sequences would be removed and only the BEV sequence would be transcribed from the CMV promoter. *Id.* The next construct describes the use of double promoter constructs (*i.e.*, having two promoters) with the BEV sequence being expressed in sense and antisense format.

20. The January 7, 1998 entry demonstrates Ms. Bernard was cloning the BEV polymerase gene into the carrier plasmid vector pCR2.1. *Id.* at pages 2 - 7. I planned to have her to clone the BEV polymerase gene into pCR2.1, which was the first step of making the BEV polymerase-GFP fusion in the vector pEGFP-N1. Once the BEV polymerase-GFP fusion was in the pEGFP-N1 vector, we planned to use a BglII/BamHI cloning strategy that would result in two alternative fusion constructs where the BEV gene sequence would be cloned in the sense or antisense orientation. Dr. Rice and I believed that once we had the two fusion constructs, we could easily insert the second copy of the BEV gene sequence in the sense and/or antisense orientation into the constructs. This was an element in making the constructs we later claimed in the '099 patent.

21. The primers BEV-1 and BEV-2 were used to PCR amplify the BEV polymerase gene sequence, corresponding to a DNA fragment of about 1.4 kilobases. We then cloned the PCR product into the pCR 2.1 plasmid vector. *Id.*

22. The January 8, 1998 entry demonstrates Ms. Bernard continued the work of January 7, 1998. *Id.* at page 8.

23. The January 9, 1998 entry demonstrates Ms. Bernard continued the work of January 7, 1998. *Id.* at pages 8 – 9.
24. January 10, 1998 was a Saturday and the laboratory was closed.
25. The January 11 and 12, 1998 entry demonstrates Ms. Bernard took steps to grow the clones obtained for the invention. *Id.* at page 9.
26. The January 13, 1998 entry demonstrates Ms. Bernard took further steps to clone BEV into the PCR2.1 and pEGFP. *Id.* at page 10. Further, Ms. Bernard describes how she validated the successful cloning of the BEV polymerase gene sequence into pCR2.1 and confirmed this by endonuclease restriction mapping. *Id.*
27. The January 14 – 16, 1998 entry demonstrates Ms. Bernard took steps to make the BEV polymerase-GFP fusion in the vector pEGFP-N1. *Id.* at pages 11 – 14. Specifically, Ms. Bernard used a *BglIII/BamHI* cloning strategy that resulted in two alternative fusion constructs where the BEV gene sequence was cloned in the sense or antisense orientation. *Id.* As previously mentioned, the *BglIII/BamHI* cloning strategy that results in two fusion constructs which could be used to easily insert the BEV gene sequence in the sense and/or antisense direction into other constructs.
28. January 17 – 18, 1998 was a Saturday and Sunday and the laboratory was closed.
29. The January 19 – 20, 1998 entry demonstrates Ms. Bernard continued the work of January 14 – 16, 1998. *Id.* at pages 14 – 16.
30. The January 21- 23, 1998 entry demonstrates Ms. Bernard used PCR to check for the presence of the BEV insert. *Id.* at pages 16 – 19. Ms. Bernard drew three diagrams depicting the location of the primers and the expected orientation of the BEV DNA sequence for each PCR product. *Id.* Unfortunately, Ms. Bernard encountered problems and the results were not as expected. *Id.* at page 17. We discussed the matter and agreed that she should try to clone the BEV polymerase gene sequence into the pEGFP-N1 again.

31. The January 21, 1998 entry demonstrates Dr. Rice used a software program to finalize his computer designs of pCR.Bgl.GFP.Bam, pCMV.Virus and pCR2.1, which I believed important to continue development. *See*, Exhibit 8 at pages 1 – 3.
32. As the January 22, 1998 entry demonstrates, Dr. Rice finalized the designs of the constructs pCMV.BEV.2, pCMV.BEVnt, pCMV.BEV.GFP.VEB, pCMV.VEB, pEGFP.BEV.1, pCMV.BEV.VEB, and pCMV.BEVx2 which I believed important to continue development of the invention. *See*, Exhibit 8 at pages 4 – 10. Dr. Rice and I were pleased with these designs. The idea we had was that once Ms. Bernard cloned the BEV polymerase gene sequence into the pEGFP-N1, we could construct pCR.BEV.2. Construction of the pCR.BEV.2 was important to reducing the invention to practice because it could be used to form the constructs we had conceived corresponding to our claims.
33. For example, in one plan we wanted to sub-clone the BEV sequence from the pCR.BEV.2 in the antisense orientation, thus producing the plasmid, pCMV.BEV.VEB. The pCMV.BEV.VEB construct comprises an inverted palindrome of BEV under the control of one promoter. As such, this construct would fall within at least claim 3. This construct is also presented schematically as Figure 14 of the '099 patent. We also wanted to make the above plasmid pCMV.BEV.GFP.VEB. This plasmid comprises an inverted palindrome of the BEV sequence under the control of one promoter with GFP as a stuffer fragment. As such, this construct would fall within at least claim 5. To make this plasmid, we would subclone the GFP from pCR.Bgl.GFP.Bam into pCMV.BEV.2 to produce pCMV.BEV.GFP. We then planned to insert the second BEV sequence in an antisense orientation. The resulting plasmid, pCMV.BEV.GFP.VEB, is presented schematically as Figure 15 of the '099 patent.
34. January 24 and 25, 1998 was a Saturday and Sunday and the laboratory was closed.
35. The January 26 – 28, 1998 entry demonstrates Ms. Bernard again attempted to clone the BEV polymerase gene sequence into pEGFP-N1. Exhibit 7 at pages 20 – 24.
36. From January 29 – February 1, 1998, the transformed cells were allowed to grow.

37. The February 2, 1998 entry demonstrates that the results of the transformation were analyzed and new ligations were set up. *Id.* at pages 25 – 26. Ms. Bernard again encountered problems. As page 25 of Ms. Bernard's notebook indicated, I discussed the results with her, and recommended she try again but instead amend the method. As such, Ms. Bernard again set up experiments to clone the BEV polymerase gene sequence into pEGFP-N1. *Id.* at page 26.
38. Ms. Bernard allowed the DHSα chemically competent cell grow on February 3, 1998. *Id.* at page 37.
39. The February 4 – 6, 1998 entry demonstrates ligations were transformed into the DHSα chemically competent cells. *Id.* at pages 27 – 30. The transformants were then PCR screened. *Id.* at page 30.
40. February 7, 1998 was a Saturday and the laboratory was closed.
41. The February 8 – 11, 1998 entry demonstrates Ms. Bernard's experiments continued. *Id.* at pages 31 – 36. We were pleased to find that Ms. Bernard succeeded in obtaining a fusion clone. *Id.* at page 33. As such, Ms. Bernard went on to sequence the fusion clone to confirm the sequence was in the clone. *Id.* at page 36. Further, Ms. Bernard hand drew a diagram depicting the location of the primers and expected orientation of the BEV-GFP sequence. *Id.*
42. February 14 – 15 was a Saturday and Sunday and the laboratory was closed.
43. Now that we had prepared the fusion clone, we were ready to take the next step. The February 17, 1998 entry demonstrates Ms. Bernard started cloning four new constructs, namely the constructs pCR.BEV.2, pCR.BEV.3, pCR.BamGFPBgIII, and pCMV cass. *Id.* at pages 37 – 39. pCR.BEV2 was a construct comprising BEV-pol that could later be used to prepare expression constructs in a sense orientation, or alternatively in an antisense orientation. As previously stated, pCR.BEV2 was an element of reducing the invention to practice because it could be used to form the constructs of our claims, including the

pCMV.BEV.VEB construct which comprises an inverted palindrome of BEV under the control of one promoter. The pCMV.BEV.VEB construct falls within at least claim 3. This construct is also Figure 14 of the '099 patent. pCR.BEV2 was also used to make the plasmid pCMV.BEV.GFP.VEB, which contained an inverted palindrome of BEV under the control of one promoter with GFP as a stuffer fragment. As such, this construct would fall within at least claim 5. Ms. Bernard also started to clone pCR.BEV3, a construct comprising an untranslatable BEV-pol. Ms. Bernard also started to clone pCRBamGFPBgIII, which is a construct comprising a stuffer for use in interrupting BEV-pol sense and BEV-pol antisense in a hairpin construct. The EGFP sequence was selected as a stuffer because it would be useful for determining whether the stuffer could mediate post transcriptional gene silencing. The GFP is flanked by the BamHI and BgIII restriction sites, so the GFP would be easy to remove. We planned to use this in our constructs that contained an inverted palindrome with a stuffer, such as pCMV.BEV.GFP.VEB discussed above. Ms. Bernard also started to clone pCMV.cass, which is plasmid pEGFP-N1 except that the EGFP gene sequence has been removed. We chose pCMV.cass as a basic plasmid expression cassette for future clones, and to later make constructs such as pCMV.BEV.SV40L.VEB, which comprises a BEV polymerase placed in the sense orientation to one promoter and another BEV polymerase placed in the antisense orientation to another promoter.

44. The February 18 – 20, 1998 entry demonstrates Ms. Bernard continued her work toward making a BEV polymerase-GFP fusion construct. *Id.* at pages 40 – 41.
45. February 21 and 22 were Saturday and Sunday and the laboratory was closed.
46. The February 23 – 24, 1998 entry demonstrates Ms. Bernard continued the experiments of the previous week. *Id.* at pages 42 – 45. Notably, she identifies the putative fusion clone (#61). *Id.* at page 44.
47. The February 25, 1998 entry demonstrates Dr. Rice designed the construct of pCMV.Lac, the diagram of which is figure 25 of the '099 patent. Exhibit 8 at page 11. Dr. Rice also designed the construct of pCMV.LAC1.pla. *Id.* at page 12.


48. On February 26, 1998, under my and Dr. Rice's direction, Ms. Bernard started to clone pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl by setting up PCR to amplify fragments for the new constructs. Exhibit 7 at page 46. As previously mentioned, I wanted to obtain pCR.BEV2 to make the constructs of our claims, including the plasmid pCMV.BEV.VEB construct, which comprises an inverted palindrome of BEV under the control of one promoter, and the plasmid pCMV.BEV.GFP.VEB, which contained an inverted palindrome of BEV under the control of one promoter with GFP as a stuffer fragment. These constructs correspond to at least claims 3 and 5, respectively.
49. On this same day, Dr. Rice designed the construct of pCMVLac1.OPRSV1.cass, the diagram of which is Figure 26 of the '099 patent. Exhibit 8 at page 13. On this same day Dr. Rice also designed the construct of pCMVLac1.OPRSVL.GFP. *Id.* at page 14.
50. The February 27, 1998 entry demonstrates Ms. Bernard continued her cloning of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at page 47. On this same day, Dr. Rice designed the constructs of pCMVLac1.OPRSV1.GFP.cass and pCMV.TYRLIB, the diagrams of which are figures 27 and 24, respectively, of the '099 patent. Exhibit 8 at pages 15 – 16. Dr. Rice also designed the construct pCMVLac.OPRSVL.GFP.TYR. *Id.* at page 17.
51. February 28 and March 1, 1998 were Saturday and Sunday and the laboratory was closed.
52. The March 2, 1998 entry demonstrates Ms. Bernard continued her work to clone the constructs of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at page 48. On this same day, Dr. Rice designed the construct of pCMV.TYR, the diagram of which is figure 23 of the '099 patent. Exhibit 8 at page 18.
53. The March 3 – 5, 1998 entry demonstrates Ms. Bernard continued her work to clone the constructs of pCR.BEV2, pCR.BEV3 and pCR.BamGFPBgl. Exhibit 7 at pages 49 – 52. Notably, Ms. Bernard confirmed the sequence of the clone on page 50 of the laboratory notebook and the PCR screened the clones on page 51 – 52.

54. The March 5, 1998 entry demonstrates Dr. Rice designed the constructs of pCMV.O.SV40L.BEV, pCMV.O.SV40L.VEB, pCMV.BEV.SV40L.O, pCMV.BEV.SV40L.R, pCR.BEV.1, pCR.BEV.2, pCR.BEV.3, pCR.SV40L, the diagrams of which are figures 17, 18, 16, 22, 6 - 8 and 4, respectively, of the '099 patent. Exhibit 8 at pages 19 - 22, 24 - 29. On this same day, Dr. Rice also designed the construct of pCR.Bgl.GFP.Bam. *Id.* at page 23.
55. The March 6, 1998 entry demonstrates Ms. Bernard ligated the amplified fragment into pPCR 2.1 to obtain pPCR2.1 EGFP. Exhibit 7 at pages 53 - 54. This was then cut with BamHI and BglII to provide a fragment, that was used to prepare a hairpin construct pBEV2.EGFP.VEB2.
56. On this same day, Dr. Rice designed the constructs of pCMV.cass, pCMV.SV40L.cass, pCMV.SV40LR.cass, pCMV.BEV.SV40L.BEV, pCMV.BEV.SV40L.VEB, the diagrams of which are figures 2, 5, 21, 19, and 20, respectively, of the '099 patent. Exhibit 8 at pages 29 - 33. On this same day, Rice also designed the construct of pCMV.BEV.SV40L.R.cass, pEGFP.NIMCS. *Id.* at page 34.
57. We were excited about the design of the plasmid pCMV.BEV.SV40L.VEB because this plasmid comprises a BEV polymerase placed in the sense orientation to one promoter and another BEV polymerase placed in the antisense orientation to another promoter. This plasmid therefore is an isolated construct of at least claim 4, and indeed corresponds to Figure 20 of the '099 patent. To make this construct, we planned to make a pCMV.SV40L.cass plasmid by sub cloning pCR.SV40L into pCMV.cass, and then insert the BEV polymerase from Ms. Bernard's pCR.BEV.2 into the sense orientation to make pCMV.BEV.SV40L.O. The BEV polymerase from pCR.BEV.2 would then sub cloned into the antisense orientation into the pCMV.BEV.SV40L.O to make pCMV.BEV.SV40L.VEB.
58. In anticipation of making the pCMV.BEV.SV40L.VEB clone, on March 18, Ms. Bernard started preparing the pCMV.cass construct. Exhibit 7 at page 66.
59. March 7 - 8, 1998 was a Saturday and Sunday and the laboratory was closed.

60. The March 9 – 11, 1998 entry demonstrates Ms. Bernard prepared larger amounts of DNA for mammalian cell transfections, including pEGFP.BEV1 and pEGFP-N1. Exhibit 7 at pages 55 – 60. Further, the MDBK cells were split in preparation for transformation on Monday, March 9, 1998. Exhibit 9 at page 1.
61. The March 11, 1998 entry demonstrates I transfected Mabin Darby Bovine Kidney (MDBK) endothelial cells with the pEGFP.BEV.1 constructs. Exhibit 9 at pages 1 – 2.
62. The March 12 – 13, 1998 entry demonstrates Ms. Bernard and I continued our respective experiments. *Id.* at 3; exhibit 7 at pages 61 – 62.
63. March 14 – 15, 1998 were a Saturday and Sunday and the laboratory was closed.
64. The March 16, 1998 entry demonstrates Ms. Bernard obtained the putative clones for pCR.BEV2 and pCR.BEV3. Exhibit 7 at page 63. On this day, I continued my transfection experiment. Exhibit 9 at page 3.
65. The March 17, 1998 entry demonstrates Ms. Bernard confirmed the clones had the proper insert. Exhibit 7 at page 64. As Ms. Bernard stated, the next experiments were to sequence clones with universal forward and reverse primers. *Id.* On this same day, I conducted kill curves for the Mabin Darby Bovine Kidney cells and started selection of constructs. Exhibit 9 at page 4.
66. From March 18 – 19, 1998, Ms. Bernard confirmed the pCR.BEV2 and pCR.BEV3 clones by sequencing. Exhibit 7 at page 67. Further, Ms. Bernard prepared the pCMV.cass construct. *Id.* at pages 65 – 66, 68.
67. The March 20, 1998 entry demonstrates Ms. Bernard continued with transformation of colonies. *Id.* at page 68. The expression cassette pCMV.cass was later confirmed by sequencing. On this same day, I continued my kidney cell transfection experiments. Exhibit 9 at page 5.

68. After this reduction to practice, I filed a patent application in Australia that was the basis for and was claimed as priority by the patent under reexamination.

69. I declare that all statements made of my own knowledge are true and all statements made on information and belief I believed to be true. I make this declaration with the understanding that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the patent.



Michael Graham

April 24, 2007
Date

EXHIBIT 1

Itaipu Nlas

(7) GEM32 Nlas

Sma Hincil

Sma Chen Hincil

Cut 200

4

Cut 300 in 200ml = 4 X

(8)

(8) JKK Nlas

Hincil 4

Cut 300 in 200ml = 4

Itaipu GUS

(9) p JKK

Kpu Sail

Sail Chen Kpu

(3)

(1)

X

Cut 100ml in 200ml = 3

(10) pBS GUS

~~YKK~~ Kpu / Sma

X

Cut 500 in 200ml = Sma

Sma Chen Kpu

(4)

(1)

(11) pBS GUS

Sma / Sail

X

Cut 500 in 200ml Sma

Sma Chen Sail

(4)

(3)

GUS → JKK

(12) GUS → JKK

Kpu / Sma Ben

Ben Chen Kpu

(3)

(1)

Cut 100ml in 200ml = Ben

(13) pBS GUS

Kpu / Ben

Ben Chen Kpu

(3)

(1)



2-9 no good

HincII site in Ula^s

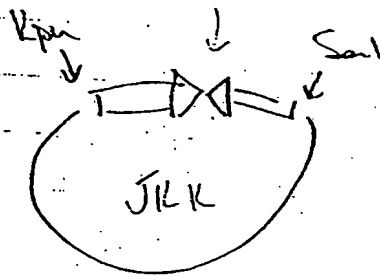
~~8~~

	A4
Vector	2ul (9)
Insert	Sma (10) Sma (11)
DDW	5
LB	2
Ligase	1

A5
2ul (12)
Sma (13)
10
2
1

Descript Hspin GUS
JKK

Ca JKK GUS



W4h

PAU, Mix High Med Low die 2, → 12
 under Mole



13x15

21→

9

2+9

were positive

Min

1 + 2 Pools of HAT7 Mts⁵
 (3 -

1-6 Pools of A2

equine fragments for double DNAs

* pSCMT

SCS4 Sma Cla H3

pMT7 Sma Cla H3

SCS4 Cut 10ul in 100ul with Sma (4)

pMT7 Cut ³ 1ul in 100ul with Sma (4)

Ligations

	J1	J2	J3
vector	2ul (1)	2ul (2)	1ul (3)
insert	10ul (4)	5ul (5)	5ul (5)
SDS	5ul	10	11
Orbiter	2ul	2	2
Ligase	1ul	1	1

script	NlaH3 → JKK	NlaH3 → PBC	NlaH3 → pBS
--------	----------------	----------------	----------------

Plate
Ka B/W

Plate Cm
B/W

Phy B/W

Mini-preps

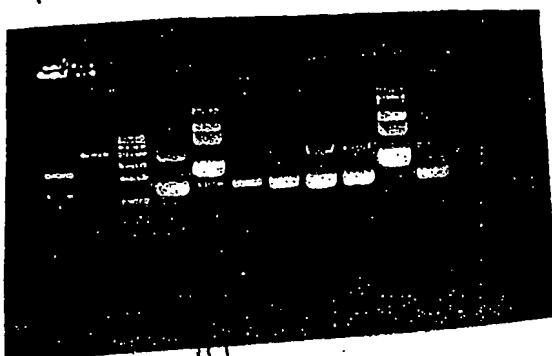
1-9 per H2

10-12 Singles per J3 - picked at plate

Cell Antis

& 6, 7 SEPI 1-9 uncut

2, 7 & 9
maybe
OK



Ligase

J4

Cut 2, 7 & 9
~~2, 7 & 9~~
Xba/H3

2ul of 2 & 7
5ul of 9

Vec 2ul 7
Insert 5ul 6
10x buff 2ul
DdW 10
Ligas 1



Cell

SEPI 10 11 12, 2, 7 & 9
H3 Xba/H3

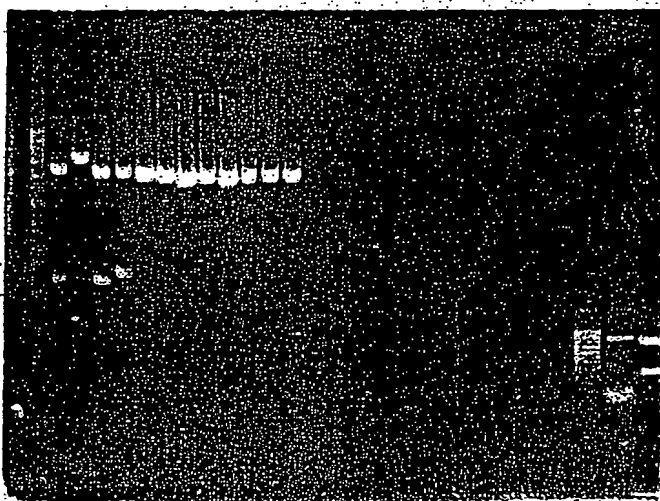
2, 7 & 9 are good!
10, 11 & 12 no DNA.

Min 1-6 whites per J3

PUC Anomolies

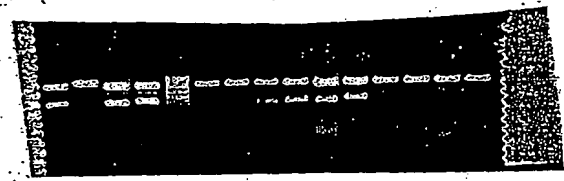
- | | | | |
|----|----------------------------|---|-------|
| 1 | 355 GUS (1) b | } | Catch |
| 2 | Rel C GUS | | |
| 3 | Sh GUS | | |
| 4 | 355 GUS | } | PLRU |
| 5 | Rel C GUS | | |
| 6 | Sh GUS | | |
| 7 | 355 GUS | } | Cold |
| 8 | Sh GUS | | |
| 9 | Rel C GUS | | |
| 10 | Sh GUS | } | Cold |
| 11 | 355 GUS PUYD | | |
| 12 | 355 GUS PUY ^N | | |
| 13 | Sh GUS 32 PUY ^N | } | PUY |
| 14 | 355 GUS | | |
| 15 | Sh GUS | | |
| 16 | Rel GUS | } | PLRU |
| | | | |
| | | | |

- 13 Sh GUS PUY^N
 14 -ve catel
 15 -ve catel



fH3 is abn
 20.8.66.

	2nd	Triphlet	SSS	20mg	100mg	200mg	300mg	400mg	500mg	600mg	700mg	800mg	900mg	1000mg	117
		Reche/2nd	Doic	Sh	200mg	100mg	200mg	300mg	400mg	500mg	600mg	700mg	800mg	900mg	
1	0.43	8.5	355	Cat - 20mg	2.4	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.6	2812
2	0.31	6.1	Doic	Cat	16.4	16.4	16.4	16.4	16.4	16.4	16.4	16.4	16.4	16.4	2601
3	0.31	6.1	Sh	Cat	65.6	34.4	34.4	34.4	34.4	34.4	34.4	34.4	34.4	34.4	51
4	0.50	10.0	355	PLAU	4	96	96	96	96	96	96	96	96	96	2333
5	0.48	9.8	Doic	PLAU	10.2	89.4	89.4	89.4	89.4	89.4	89.4	89.4	89.4	89.4	1356
6	0.27	5.3	Sh	PLAU	75.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	123
7	0.29	5.8	355	- Cold -	6.9	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	3587
8	0.29	5.8	Sh	- Cold -	6.9	31	31	31	31	31	31	31	31	31	115
9	0.38	7.6	Doic	PUY	13.2	46.8	46.8	46.8	46.8	46.8	46.8	46.8	46.8	46.8	2200
10	0.26	5.1	Sh	PUY	78.4	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6	88
11	0.85	17.0	355	(PUY)	2.4	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.6	97.6	4215
12	0.50	10.0	355	(PUY)	4	96	96	96	96	96	96	96	96	96	4355
13	0.42	8.4	Sh (m)		47.6	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	53.3	133
14	0.36	7.2	ne (Somy)	m	55.6	44.4	44.4	44.4	44.4	44.4	44.4	44.4	44.4	44.4	- 28
15	0.39	7.8	ne (Somy)	m	51.3	48.7	48.7	48.7	48.7	48.7	48.7	48.7	48.7	48.7	- 29



1-6 43
 H3/P1
 P2/P1

2+3 both good.

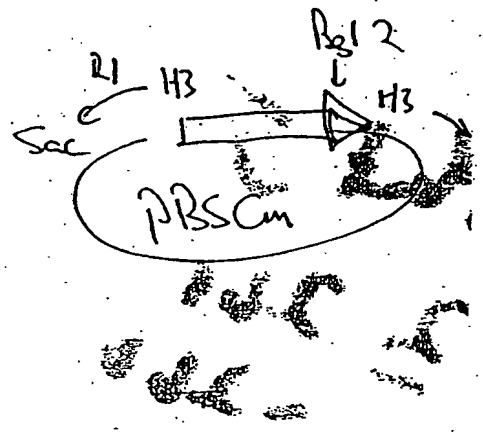
Control "Cold" both the same cri.

355 CUS

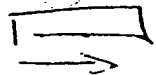
Cell 2=3
 3=4

PLAU CUS

PUY CUS



CHECK

21 Sac Kpu Sna Re H3
JS ~~21~~ 

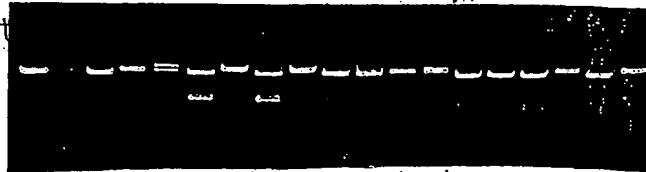
119

Mimis 1-6

J1 Cut ~~21~~ H3 (2) 104 H3. JKK
Rg 2/21 (2)

7-11

JS Cut 21/H3



Suspect these O.C

J1 ?

JS Fine

Grow
3 as
stock

Over 3 or 4
as separate
stock

Obvius!

- Set Bam (blind) /H3


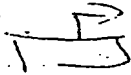

GEM 92 Nsi (blind) /H3

Ger ADT SC4 ADTSC4

- JKK SC4 H3/Re

Double header

ADT 7 H3/Re

-   

JKK SC4 Re (blind)
H3

ADT 7 Re (blind) /H3
into ~~SC4~~ Cb (blind)

Cb (blind) /H3

Col DISPD 10, 11, 12, 13, 14, 15

Ligation



	J6	J7
Vec	10 (11)	10 (15)
Inert	10 (10)	10 (14)
DNA	1	1
Water	2	2
Ligase	1	1
Deoxi.	1	1
	15 16	15 16

Next

None of these were any good start

Repeat (10) (12) (14) (15) (16) (17)

Also HAT 7 H3 (18)
pJRK Wla H3 (19)

10
13
14
15
1-30

Xba Sal

- 1) Sma Cla H3 Cut Sal i Sma (4)
- 2) pBSCu Cut Sal with Xba
- 3) St. ass Cut Sal with Sac
- 4) Sal i H3

Minis

1, 2 + 3

Sal/Kpn (4)

4

Not

(3)

4637 (Rob)

Ben (3) Cut Tail in 100 w

3553'

Not/Ben (3)

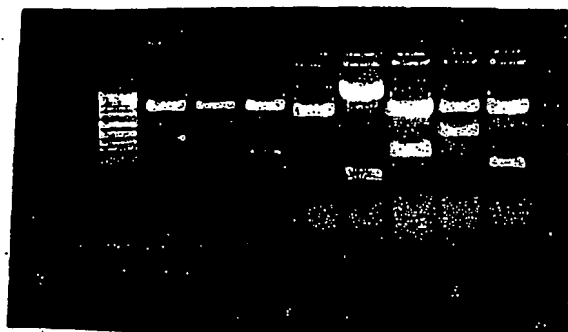
Check

355 pu

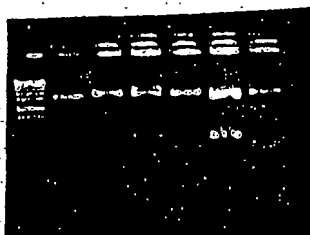
Not/Ben (3)

pBS SC4

Not/Ben (3)



1-6



← CHECK 6

Construct

SC4 cassettes.

(1) ADI 7 Δ Not Cut Sml \bar{c} Not I (3) 10^{40} (2) Not che Cla pBC. SCS4 (3 che 4) 10^{40}
Cut Sml(3) Not che Cla pBC. 35S 3' (3 che 4) 10^{40} (4) Not/Xho BC. SCS4 Cut Sml in 3 10^{40} (5) Not/Xho BC. 35S 3' Cut Sml in 3 10^{40}

(1) + (2) + (3), (1) + (4) + (5) -

Intermediate \rightarrow  +   (6) Δ BC LT22 & Sml Ban/Cla in 2 10^{40} Then blunt \bar{c} TH + recve(6) 8 Tml DDW 1.45
10 ml 10x buffer
1 ml Sml SWT's
2 ml T4 ligase

	Q1	Q2	Q3
Deck	2ml (1)	2ml (1)	1ml (6) blunk
Insert 1	4ml (2)	4ml (4)	-
Insert 2	4ml (3)	2ml (5)	-
Or ligase	2ml	2ml	2
Ligase	1	1	1
DDW	7	9	16

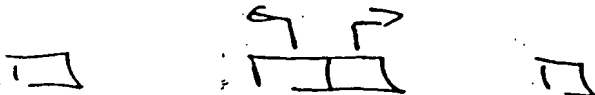
ADI7.SCS4

ADI7.SCS4

 Δ Ban/Cla
pBC.LT22

2 ?

(C)



(7)

Apa I / B1

BC. LT22

(2)

Cut Sml in buffer 2

2¹⁰

(8)

Not I / B1

BC. 355 2'

Cut Sml in buffer 3

2¹⁰

(9)

Apa I / Not I

pBS

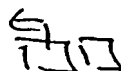
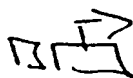
Cut Sml Apa I in buffer 2

2¹⁰

Then cut Not I in buffer 3

by B1W x 2

(10)

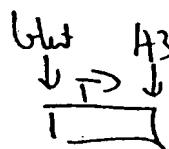


(10)

JVK SCS4

Cut Sml

Kpn I

(1)
~~(Gluat)~~ / H3

* Then blunt

Then H3 (2)

87 B1W
line 1
3ue H3

A. 12

(11)

Hinc II / Hinc III

Cut Sml buffer 2

2¹⁰

PBC. LT22

(12)

pBS

AAT 7

Not (blunt) H3

AAT 7

Not

Sml in buffer 3

2¹⁰

* Blunt

H3 in buffer 3

(19) 4639 Bam / Sma

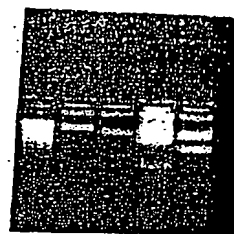
Cut Sma Bam / Sma (4)

(20) JKK Mla^S Bam Sma / Bam (4)

Cut Sma Bam / Sma

Analytical cuts

- 4639 Ba
- 4639 Sma



(19) + (20)
1000

- JKK Mla^S H3
- H3 / Rg12
- Rg12
- Sma
- Bam

(17) + (18)

Q & 10

Vec 100

Insert 5

DDW 1

100bp

Ligase

13 15

(21)

~~PBC~~
~~PBS~~ ~~PK4~~ H3

PBC PUV H3

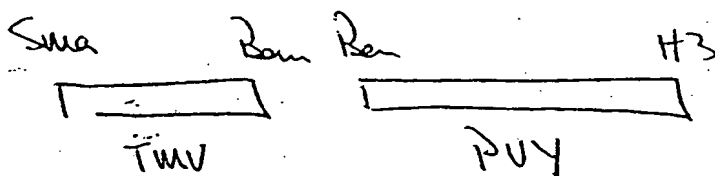
- Bam
- Bam / Rg12
- H3

(22)

Clone PUY TMU pairs

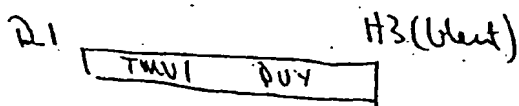
(1)

(A)
picks up



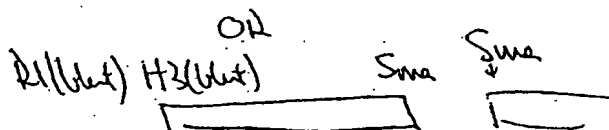
into Sma/H3 JKK
Both aries behind pBC-GUS

(a)

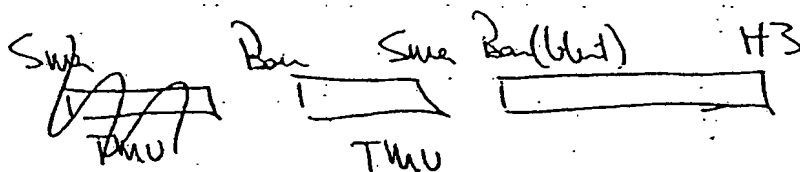


into A1/Sma pBC-GUS

(b)

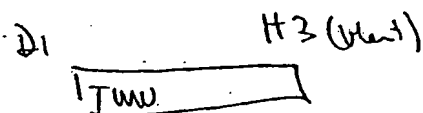


(2)



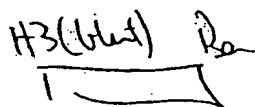
into Bam/H3 JKK

(a)



=> A1/Sma pBC-GUS

(b)



=> A1 (blest) Ba pBC-GUS

(6) JKK Bar / Sue

Cut 10ml Bar / Sue 4

10 JKK
10 B4
74 DDu
3 Bar
3 Sue

~~(7) JKK~~

(7) 4637

Bar

Cut Sue Bar 4

8
5 4637
10 10x3
82 DDu
3 Bar

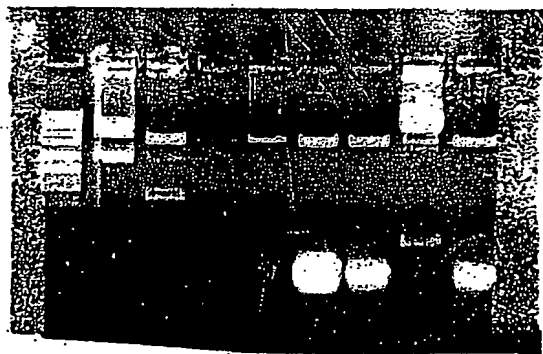
(8)

JKK

Bar

Cut 10ml Bar 4

10 JKK
5 10x3
82 DDu
3 Bar



1315 PL 1315

(9) ~~PBS 25 02 31 35 50~~ PBS

Cut Sma ————— Sma

Not/Apa
Not (3)

then Apa

(10) PBS LT 22 Sca

Cut Sma ————— Apa/Sma

Sma (4) Apa/S
then Apa (4)

(11) PBS ccs 3' 35 pro

Not/Sma Sma (4) Not

then Not then

(12) PBS 2x3's

Cut Sma

Pst (3)

Then blunt
H3

X

(13) pBC-SC4

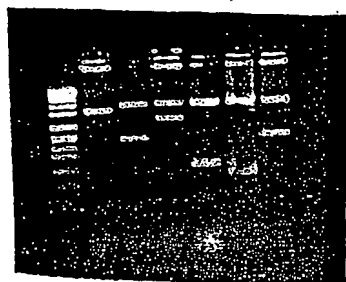
H3 (2)

Blut

Not

X

(14) pBC-35s pro Not/Al Not/Al (3)



9 10 11 12 13 14

(12) is too good! Picked up a Δ or H3 in one of the 3's
Must be in LT22! ??

Ligali

25

Vector

1ul (9)

Inner1

3ul (10) or 5ul (11)

DDW

8

10x buffer

2

Ligase

1

Descript

1111 PL 1111

Repeat the PUY: TUV ligation using vectors back
 2x vector - half insert. ~~024~~

	X1	X2	X3	X4	
Vector	4ul (4)	2ul (5)	1ul (6)	4ul (8)	
Insert	1ul (1) 1ul (2)	5ul (3) 0.5ul (1)	2ul (1) 2ul (1)	2ul (7)	
W/buffer	2	2	2	0.5	
DDW	11	9.5	14	11	
Ligase	1	1	1	1	

Descript	PUY TUV (1)	PUY TUV (2)	TUV (2) TUV	TUV (2) TUV	
	JLK	JLK	JLK	JLK	
Plate	K B/W	K B/W	K B/W	K B/W	
ClS	11	11	11	11	

Diagrams

Gene CIVE + 1/2 E
into C4 can

Section

ADT7. SCS4 DU/Rom

DI/Swa

Insert

- Xho(Blunt)/Rom (3)

(1) (2)
Bam

- Xho(Blunt) DI (4)

Cur Sul DU/Rom (3)
ADT7. SCS4

(2) Cur Sul DI/Swa (4)

(3) Cur Sul Bcl. CIVE Xho
Trans. Rom 13

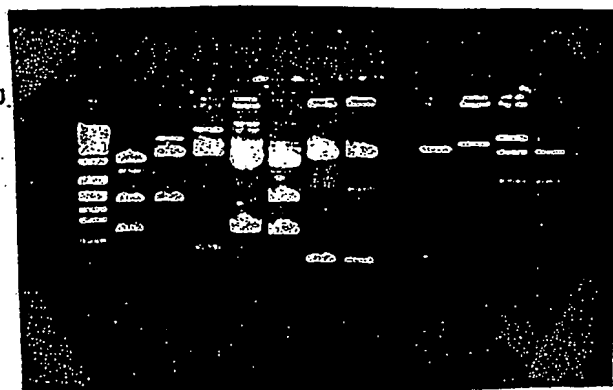
(4) Cur Sul Bcl. CIVE Xho
Trans. Rom 12

(5) pBC-GUS Bam HI (3)

(6) 4637 Bam H.I. (3) 5' 3' 182

Cells

12 3 4



(1) Something is wrong

(1) + (3) leave it

(2) + (4)

G1

G1

5 6



Vect 4400 2ul (2)

2ul (5)

Invert 5ul (4)

2ul (6)

DDW 10

13

Ligase 201

1

10x buffer 2

2

Connect

pBC

SCS4-GUS & -sense

← This Ben pBC-GUS.

Plate

Amp

Am

S/S

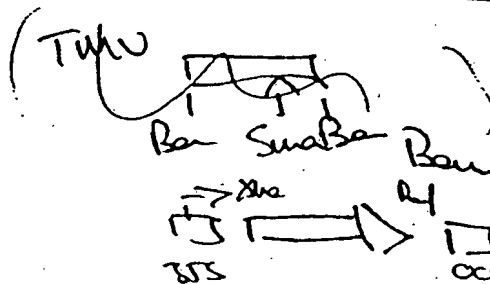
Am

Amp

168

Z1

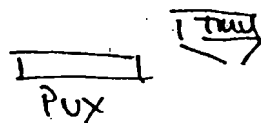
1-12 ADT7. CUS



Z2

JKK. PUY. Tnuv

B-24



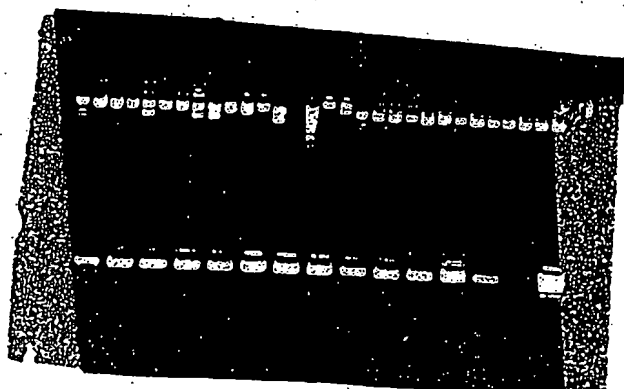
Z3

PBC. CUS. Tnuv

25-30



	Z1	Z2	Z3
Vector	2ul (8)	2ul (8)	5ul (4)
Insert	4ul (7)	4ul (5)	3ul (5)
DDW	11	11	9
WxLigase	2	2	2
Ligase	1	1	1
Plate	Amp	Ban	Cin
Select	Cin	Amp	Amp.



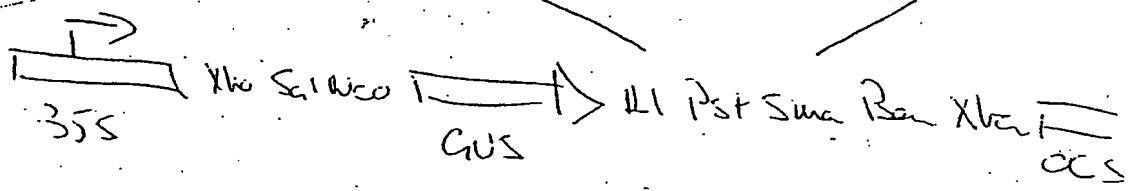
Z1 : 8 or 11
Z2 : 21
Z3 : 21

35 is probably
we good!

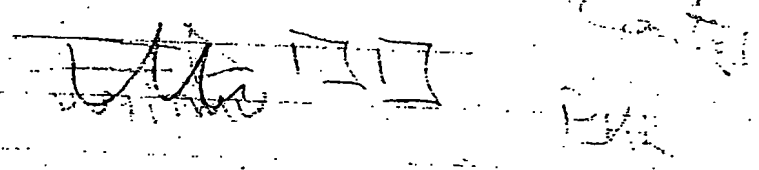
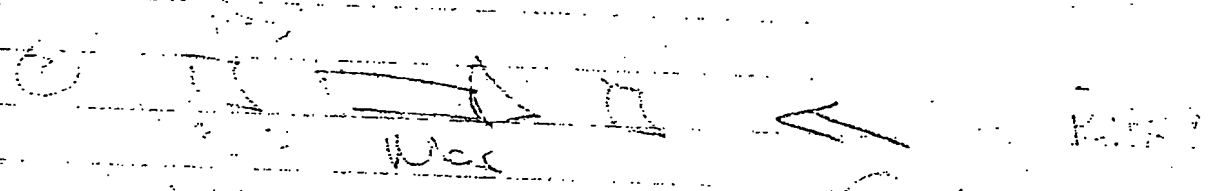
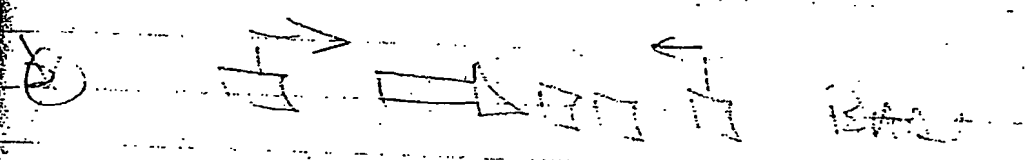
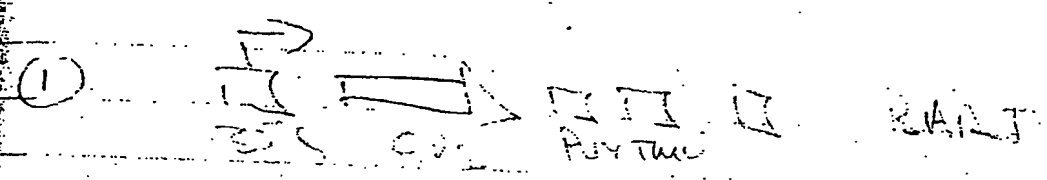
21

R1 Sec Kpn Sum Ave Ben

HF3 (blue) 169
TWO POY



For maintenance of a cusk



Check 355 Wco

Cus Sec Wco

For maintenance of a cusk



①

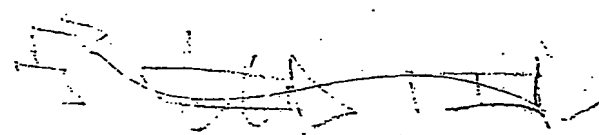
- One
- One

②

One

22 # 34

③



ABT

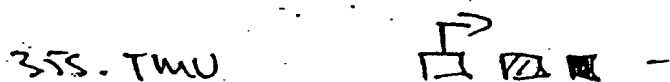
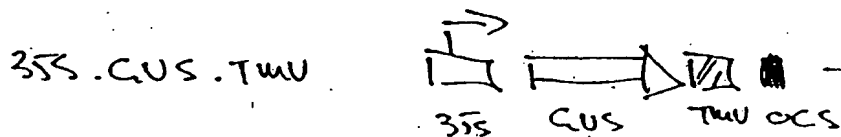
③

One ABT 21H3 62

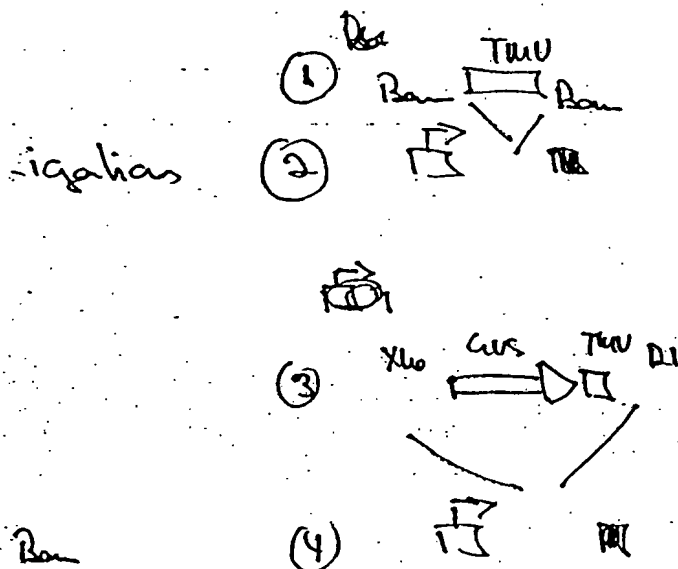
One ABT 21H3

CUS . TMU for a CUSb plants. 175

Wat



Have (Xho) CUS TMU (DI) pBC Z3 . 35
pBC . CUS . TMU



4637 Ban

ADT7 Ban

(9)
(10)

Do 33 + 35

Z3 . 35 Xho / ~~ADT7~~ Not (blunt)

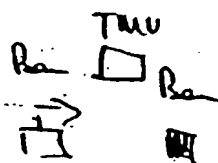
ADT7 Xho / ~~ADT7~~ ^{Sure} Not (blunt)

- (1) 10 Ban 4637 Ban (3)
- (2) 10 ADT7 Ban (3)
- (3) a 10 Z3 - 33 Not then blunt then Xho (3)
- (3) b 10 - 35 Not then blunt then Xho (3)
- (4) 10 ADT7 Sure then Xho (4)
- (5) 10 pBC . CUS . TMU Ban (Z3 - 33) (3)
- (6) 10 ADT7 Sure then Xho (4)
- (7) 10 TMU Ban
- (8) 10 ADT7 CUS Ban Z1 # 4

4 Ligation

181

A1 ADT7-TMV

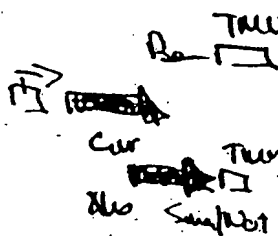


pRC.CUS.TMV

Ba (5)

ADT7 Ba (2)

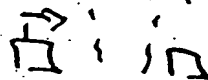
A2 ADT7.CUS.TMV #1



pRC.CUS.TMV

ADT7.CUS Ba

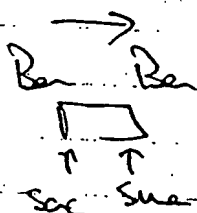
A3 ADT7.CUS.TMV #2



pRC.CUS.TMV

ADT7 Xho/Sma

A4 BAST.CUS



Not BamHI

(10)



(9)

	A1	A2	A3	A4
Vector	2ul (2)	2ul (8)	3ul (4)	3ul (9)
Insert	4ul (5)	4ul (5)	5ul (30)	4ul (10)
DDW	11	11	9	10
Water	2	2	2	2
Ligase	1	1	1	1

Description	ADT7-TMV	ADT7.CUS.TMV #1	ADT7.CUS.TMV #2	BAST.CUS
Plate	100	100	100	100
Concentration	Cu	Cu	Cu	100

1-12 A1

13-30 A2

31-36 A4

Cut

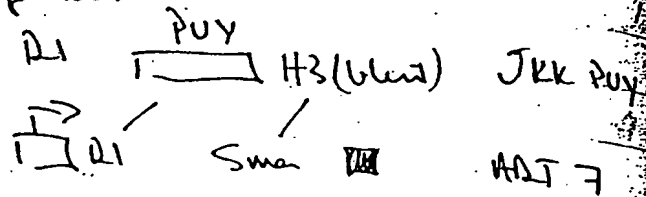
Not I

Not RI

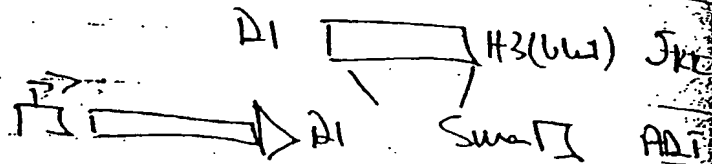
Not I

2 Ligation

A5

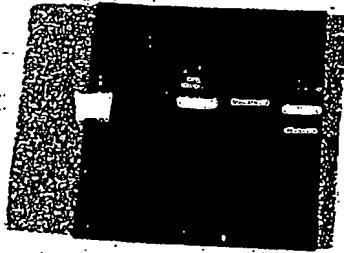


A6



DNA

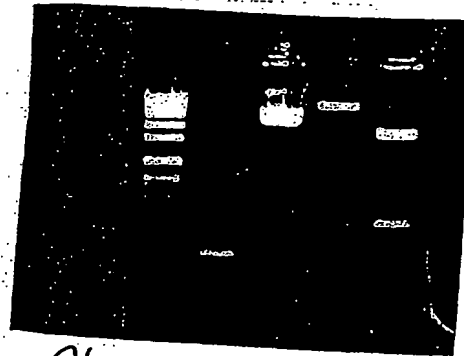
- 1 ADT7 ~~DI~~ DI/Sma
- 2 ADT7. CUS DI/Sma
- 3 JKK PUY Ban/H3 DI/H3 (blunt)



A5

1ul 1
4ul 3
12
2

ADT7 PUY



A6

2ul 2
4ul 3
11
2
1

Vector
Insert
DDW
10x buffer
Ligase
Descript

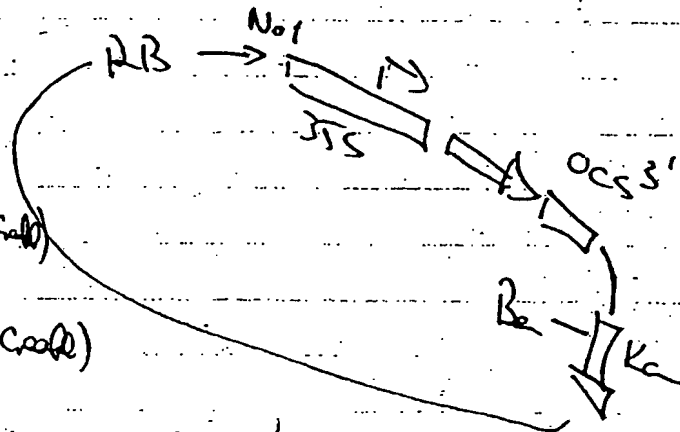
Plate
(Select)

Shot Thursday

(23)
(27)
(30)LP x 8 LP⁻ x 2

Tuesday (16) (19)

3xPs

~~BART. EUS~~
~~BART~~orientation of all
brines is:

BART. THU Ligi 1 #1 (p31 coll)

BART. CUS (#33 p30 coll)

BART. CUS. THU Ligi 2 #5 (p31 coll)

9/9/94

Skin

16

3 pieces

(19)

3 pieces

+ 2 x 355

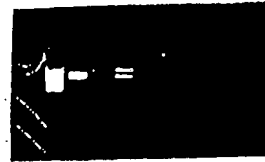
+ 2 x 180 -ve cat

Cut

20

(11) iCUS Sum/Bl (4)

(12) pRC Bl/Bl (3)

(13) ~~ieuzt~~ JVK PUY H3Kul Ba (3)

10 OK

11 partial!

12 didn't cut

(14) mCOOS Ba (3)

→ 13 no Dist

Regen → 14 didn't cut

Regen

PUSH TITRANT

Grid sampled

(1)

(3)

(2)

This is the way it's operated
Sheet

MUNA BO

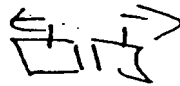
(1)

(2)

(3)

Further cloning

DP2



Intermediates

- ①
- ②
- ③

pBC-SC4

Not ID1

CutSue buff

pBC-355

Not ID1

JKK R1

④

pBC-SSO3

HS

Pst

3 ✓

Blunt

HindIII

⑤

pBC-SC4

Bam

3

✓

Blunt

HindIII

⑥

pBC-7

Bam / Xba

2

✓

Blunt

⑦

pBC-SC4

Not ID3

Not

(3)

✓

200 6 17

Blunt

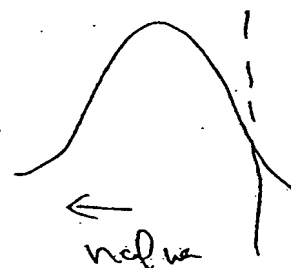
H3

(2)

Blunt



Ab



Monday

Creab 4 Tolence TF

- B.S.
- Petra SINC
S7WC
- M.C. BART. TMU

Tuesday

4 x a CUSb lines

Neil

- BART. CUS
- BART *

} 2 plates each

Wednesday

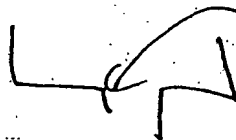
Neil

Dilution series into W38

Silver ink

Switch on ?

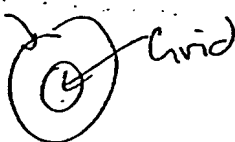
(1)



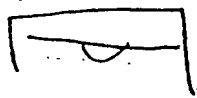
Small line

Tighter with
brush

(2)

Cold
Side

(3)

Seed on
top

Down

Upside
down

(3) RI/Pst PBC-2x3's (3) ✓

(4) RI/Pst PBC-2 penates (3) ✓

(5) Macroos SEM3E. PUY. TUV. (4)

~~Pst~~ Kpn (10)
~~Sma~~ Blunt

(6) PBC. CUS (4)

Pst
Sma

PBS SSO 3'
Sma Ben Spe V

(7) Macroos

Ben (3)

1] Pst Sal C6H3 Pst
SSO

(8) PBC 2x3's 2V/R1

(9) PBC 2 penates Sma/R1

(b) pBC-SC4

Not (3)
Hind III (2)
Blunt

(7) ADI7

Ban/Xba (2)
Blunt



Ligase

	b	18	12	
	X1	X2	X3	X4
Vector	2ul (1)	2ul (3)	2ul (5)	1ul (7)
Insert	-	2ul (2)	4ul (4)	6ul (6)
Orbital	2ul	2	2	2
DPW	15	13	11	10
Ligase	1	1	1	1

Desc	2 pos ADI7	pBC-SC4	pBC-GUS	DP3 (C)
		SC4	Puv.TM	
Plate	Kan	Cm	Cm	Amr
C/Selet			Amr	Cm
Con	Not	Clb	Pst/A	
	3	2	3	

Preparative cuts

X	10	pBC 2x 3's	Du 121	3
X	11	MCO6	Sma 121	4
	12	pBC sch Bam		3
		blunt		
		Sal		3
	13	pBC SSU3'		
		Pst		3
		blunt		
		Sal		3
	14	pBC sch	Not	3
		Noty/B	Hind III	2
		blunt	blunt	
	15	ART 7 Bam / Xba		2
		blunt		Dec

Ligation

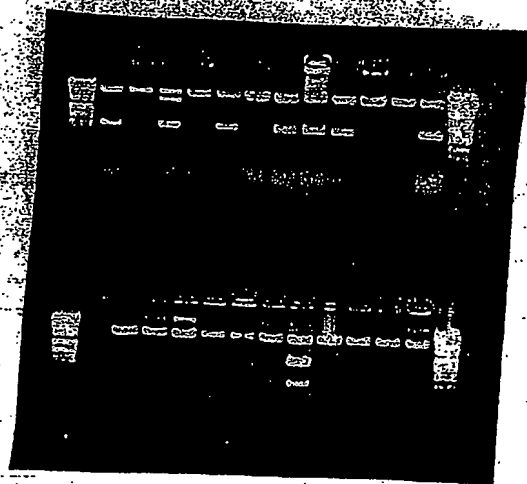
	X10	X1
Vector	2ul B	1ul 15
Insert	4ul 12	4ul 14
DDW	11	12
Wash	2	2
Ligate	1	1
Descript	pBC SSU3'	15 15
Probe	Can	Prop
Check	-	Can

B7

Crew #1 as PBC.GUS.PUY.TMV

B5

Crew #2 as DP3 ! hp

B1C
H3 HK4

Mun 1-6

7-18

B9

B8

Cla (1)

Sine (4)

Reds 1-6



Reds 10 & 18 have
B8 which is
DP2

are the following vectors

V1 F017 Sma / Xho (4) (I1)

V2 DP2 Hnd III (2) (I1)
Mco10 Blunt
Xho (2)

V3 DP2 Spe (1) (I2)
Mco10 Blunt
Pst (3)

V4 DP3 Sal (3) (I2)
Mco11 Blunt
Xho (2)

V5 DP3 Spe I (1) (I3)
Mco14 Sma (4)
13 sub
12 sub

(1) pRC SC4 Not (3)
Blunt
Sal / Pvu2 (3)

(3) pBS
SSU3' acc 3'

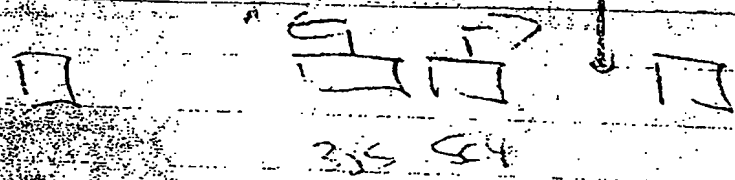
(2) pRC SSU3' Pst (3)
Blunt
Sal (3)

Pst (3)
Blunt
Sal (3)

(4) pRC SC4
Not
Blunt
Sal

DP3

Swa



Both cons
Du?

both cons

Get/Ret blut

Get

* Mapping

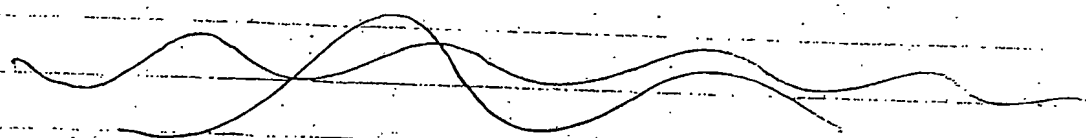
DP3

Xho
Xba

PRE SSU3'

- (Huc) II

PRE 2x3's } Xho
PRE SSU3' } Xba



VVI

Monday

X Cloning into DP1

UDPI 1 DP1 Xho (2)
(Mcoob) H3

(2)

UDPI 2 Xho (2)
BlnI

UDPI 3 Xho (2)
HindIII

UDPI 4 Xho (2)
Xba

UDPI 4 Xho (2)
EcoRV

IDPI 1 pBC.gux.TMV. PUY (Mcoob)

SacI (3)

HindIII (2)

IDPI 2 Mcoob

EcoRI (2)

BlnI

HindIII (2)

IDPI 3

Mcoob

Xho (2)
Xba

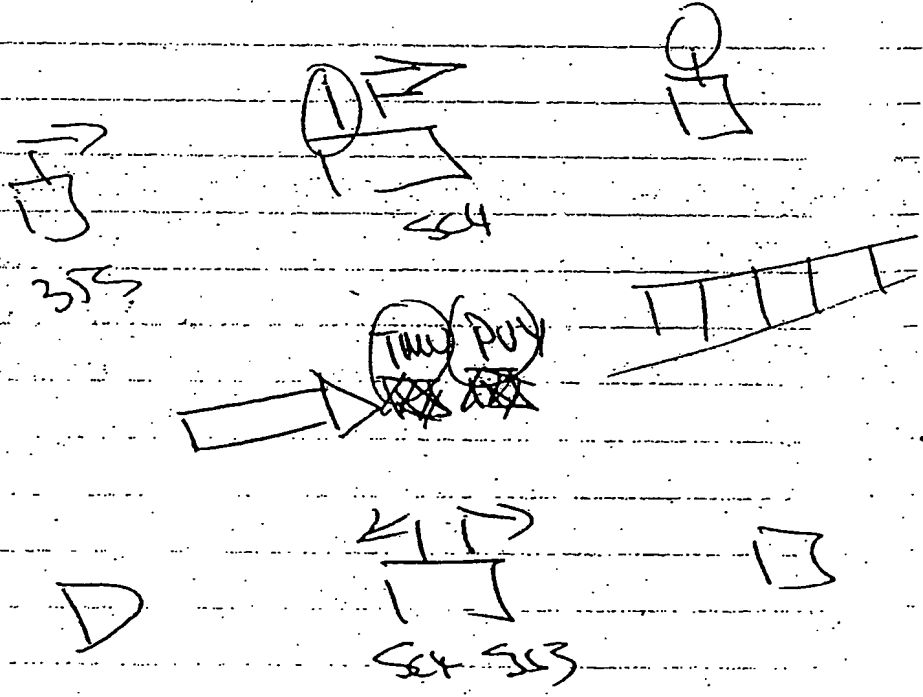
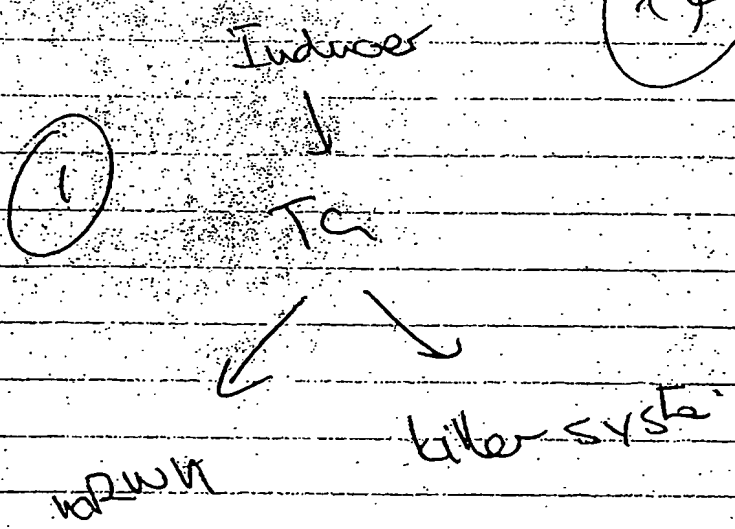
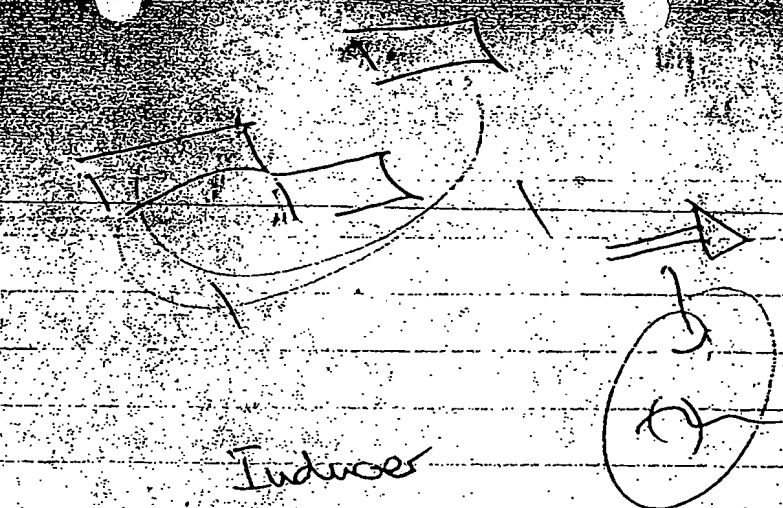


EXHIBIT 2



PLANT
INDUSTRY

CSIRO Division of Plant Industry
Institute of Plant Production and Processing

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GPO Box 1600
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Australia

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Black Mountain, Canberra ACT
Tel (06) 246 4911 Int +61 6 246 4911
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FACSIMILE TRANSMISSION

John Slattery
Davies Collison Cave
GPO Box 4387QQ
Melbourne VIC 3001

Dear John,

Re: Patentability of new approach to gene inactivation

Attached is an outline of an idea that Mick Graham has regarding antisense technologies. We would like to get your opinion as to whether we have enough basis to file immediately for a provisional patent.

The concept emerges from the fact that introduced genes (transgenes) are capable of suppressing expression of endogenous genes when the transgenes are in either the normal or reverse orientation. The reverse orientation approach has become known as antisense; the normal orientation is becoming known as sense co-suppression. Both antisense and co-suppression are the subject of existing patents.

It has been thought that antisense works by the binding with the opposite endogenous mRNA sequence thereby preventing translation of the message, but there has been no definite proof that this is the method that operates *in vivo*. On its own, such a mechanism cannot account for the co-suppression that occurs when a transgene is inserted in the sense orientation.

Mick's hypothesis is that, in some instances, sense transgenes become inserted in chromosomal positions where a partial (or even complete) antisense transcript is produced, thus leading to a similar sense/antisense mRNA hybrid molecule forming. Further it is suggested that it is not the passive formation of the mRNA hybrid alone that prevents translation (expression), but rather this hybrid induces an endogenous mechanism that destroys such hybrid RNA molecules in a sequence specific way (perhaps the action of a ribonuclease?). If this proves to be the case it leads to a number of new ways of exploiting this phenomenon, some of which are outlined in the attachment.

A range of scientific evidence now seems to be pointing strongly in the direction of this theory and Mick feels that it won't take long for other research groups to come to similar conclusions (if they haven't already done so). Thus there is a strong sense of urgency about getting as early a priority date as possible. It should be possible to prove or disprove the hypothesis within the 12-month period. We should be able to produce the transgene constructs outlined in the attachment and determine whether they have the postulated gene suppression effects in transgenic organisms.

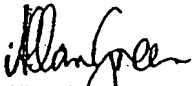
My view is that this idea synthesises existing and emerging knowledge of both "antisense" and "sense" suppression of gene expression into a novel hypothesis as to the fundamental mechanism leading to these effects. If this proves to be the mechanism operating in such transgenics, then it leads to a range of novel

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approaches to down-regulation of gene expression that would appear to be advances on (or at least
...enting around") standard "antisense" and "sense" approaches. Is this inventive enough (if proven by
subsequent work) to justify a patent application, and should we go ahead with a speculative provisional based
on the idea because of the potential value of this technology?

I would appreciate receiving your opinion on this as soon as possible so that we can proceed to prepare
more detailed information for a provisional patent application if it is warranted.

Sincerely,



Allan Green
Principal Research Scientist

cc Mick Graham
 T.J. Higgins
 Pat Walsh
 Jim Peacock

Rationale

We consider the mechanism of "anti-sense" and "co-suppression" are identical. Both involve a change in the function of the transgene, from producing a normal mRNA to interacting with endogenous factors, which results in the transgene mRNA functioning as a sequence specific RNAase.

This "state switch" requires an interaction between the transgene and the endogenous homologue - this interaction probably involves hybrid formation between the endogenous mRNA and the transgene.

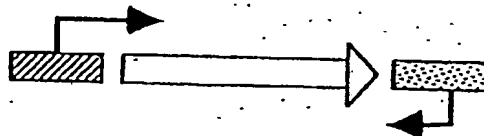
The difference between a weak and strong phenotypic effect in different transgenic lines results from differences in the propensity of the sense or anti-sense transgene to switch states. The differences in sensitivity is possibly a consequence of transgene integration into existing transcriptional units.

Improving existing strategies to create strong phenotypes

In order to obtain consistently strong phenotypic effects, constructs with a stronger propensity to switch states can be designed. These will produce RNAs which will form double stranded hybrids thereby resulting in an increased likelihood of inducing the state switch.

Possible constructs envisaged are shown below. The shaded boxes with the filled arrowheads represent promoters, the arrow shows the direction of transcription. The open boxes with open arrowheads represent coding sequences, the arrow representing the sense orientation of the gene.

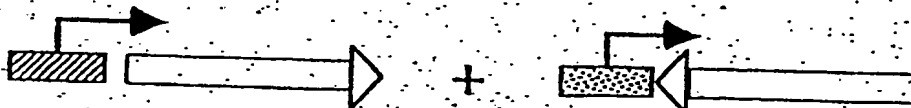
Case 1: Two promoters drive expression of the same transgene in opposite directions resulting in formation of complementary RNAs that can form hybrids, hence switching states.



Case 2: A single promoter drives expression of an inverted repeat. The transcript can then form a hairpin hybrid hence switching states.



Case 3: A sense and anti-sense construct are introduced into the same cell either as a consequence of a sexual cross or by super-transformation. The two separate transcripts can then form a hybrid hence switching states.

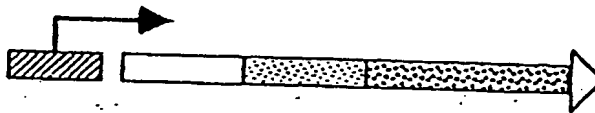


Another implication of this model is that the use of hybrid RNAs will permit the design of constructs that will inactivate multiple genes. Once a gene switches state the sequence specific RNAase activity will be a property of the complete RNA sequences of the switched transgene. Thus any endogenous gene that contains sequences homologous to sequences in a switched hybrid transgene will be suppressed.

Such constructs might be used to induce the state switch more rapidly using a more abundant or constitutively expressed RNA as the endogenous inducer.

In addition single constructs consisting of a promoter driving hybrid sequences should inactivate expression of all endogenous genes present in the hybrid RNA. This might for example permit the design of single gene constructs to protect against multiple viruses.

Case 4: Different portions of the hybrid RNA are shown by different shading. A single promoter driving this construct (or constructs designed as for cases 1, 2 or 3) should suppress the expression of all endogenous genes whose sequences are present in the hybrid. The state switch can be induced by interaction with only one sequence, but the consequence will be suppression of all sequences present in the switched transgene.



Expected outcomes

- These strategies should permit the design of constructs that will be more effective at creating transgenic lines with strong suppressed phenotype. For example, typically 1 in 10 transgenic lines for a given construct will display a strong phenotype. The use of constructs with a higher propensity to switch should result in a higher proportion of transgenic lines showing strong phenotype.
- Intermediate phenotypes resulting from anti-sense or co-suppression are almost certainly phenotypically chimeric and intrinsically unstable. These constructs should result in a lower degree of such chimerism and an increase stability of phenotype.
- For case 4 novel strategies can be designed to inactivate multiple genes. For example hybrid RNAs consisting of sequences from different viruses should protect against all those viruses once the state-switch occurs.
- Such constructs should be useful in mammalian systems.

EXHIBIT 3

PROVISIONAL PATENT SUBMISSION

Introduction

This invention describes a novel approach to creating gene "knockout" phenotypes in plants and animals by transgenesis. The invention will permit the efficient creation of plants and animals with little or no expression of the targetted gene or genes, including the creation of viral resistant or immune plants or animals.

This is currently achieved using anti-sense or co-suppression. Both methodologies seem to act by the same mechanism, namely activation of an endogenous sequence specific RNA degradative system. Activation of this endogenous system usually requires a "trigger", namely appearance of the target mRNA or viral RNA in cells of the organism.

This invention describes the design of transgenes which are either more sensitive to the trigger, or trigger themselves. Engineering plants and animals with these constructs will result in:

- A higher proportion of transgenic organisms expressing strong "gene knockout" phenotypes. Only a low proportion of transgenics produced using anti-sense or co-suppression display strong phenotypes, most display sectoring, namely areas of tissues or organs are viral immune or do not express the target gene, whilst adjacent areas express target genes normally or support normal levels of viral replication.
- More stringent "gene knockout" phenotypes. For constructs with more sensitive triggers, activation of the RNA degradative system will therefore occur earlier in development with less inducer. Thus with viruses for example little if any replication will occur before immunity is induced, whilst for "gene knockouts" little if any expression of the target gene will occur before the system is induced. For constructs that trigger themselves no inducer will be required, resulting in true viral immunity or "gene knockouts".
- Phenotypes will be more stable. Both anti-sense and co-suppressed phenotypes are unstable, the phenotype can revert during development resulting in complete reversion or sectoring.
- Strong phenotypes will be obtained in heterozygotes or primary transgenics.

CONCEPT

1. The invention is based on recent observations indicating that anti-sense and co-suppression function by activating an endogenous RNA degradative system.
2. There is a trigger which activates of the endogenous RNA degradative system. The triggering event is usually induced by the presence of the target RNA, either the virus or the targetted RNA.
3. Rare transgenic insertions trigger themselves, presumably through interactions between repeated inserts.
4. Novel transgene design can permit:
 - Transgenes with an increased sensitivity to trigger early in development.
 - Transgenes that trigger themselves.

5.

DEMONSTRATION

1. Tobacco were transformed with the constructs shown in Figure 1:

A. GUS PVY/TMV fusion driven by the 35S promoter

B. An anti-sense GUS PVY/TMV fusion driven by the SCSV 4 promoter.

C. A "double promoter construct" (DP2): containing two transcriptional units, a PVY/TMV fusion driven by the 35S promoter and an anti-sense GUS PVY/TMV fusion driven by the SCSV 4 promoter.

D. A "double promoter construct" (DP1): containing two opposing promoters driving expression of the GUS PVY/TMV fusion.

2. Primary transformants were infected with PVY using manual inoculation.

3. Symptom development was monitored visually, and PVY replication was monitored using an ELISA assay

4. Results indicate that a much higher proportion of plants transformed with construct C were resistant to PVY (Table 1).

EXHIBIT 4

MAPPING TRANSGENE ACTIVITY IN PLANTS USING VISIBLE PHENOTYPES

Michael W. Graham, Ming Bo Wang, Bob Furbank, Steve Trevanion, Simon Robinson,
Richard Forster, Paul Keese and Peter Waterhouse

CSIRO Division of Plant Industry, GPO Box 1600, Canberra ACT, 2601

Introduction

Transgenic plants are relatively easy to create. Typically to analyse transgene expression many primary lines are generated but only a few showing the desired expression pattern or phenotype are selected for more detailed analysis; lines showing unanticipated expression patterns are frequently discarded. Much valuable information regarding transgene expression is lost during this culling process, in particular evidence refuting tacit assumptions that transgenes normally behave in a uniform, predictable Mendelian fashion.

Transgene expression is usually monitored using biochemical or molecular assays. However the information gained from such approaches can be of limited value, especially when spatially or temporally regulated processes are considered. For this reason visual marker genes such as GUS or β -galactosidase are frequently employed. Marker genes can provide more detailed information on gene expression and since assays are quick and simple, large populations of transgenic lines can be more readily analysed.

In plants, transgene expression itself sometimes creates visible phenotypes. Figure 1 shows an example of a single leaf from a transgenic *Flaveria bidentis*, a tropical C4 plant. This plant was transformed with a construct designed to inhibit expression of the key photosynthetic enzyme pyruvate phosphate dikinase (PPDK). In green tissues of this leaf both PPDK activity and photosynthesis are normal. In contrast PPDK activity is not detectable in the yellow areas, which are consequently non-photosynthetic. If this whole leaf were ground up and assayed conventionally, PPDK protein levels, enzyme activities and mRNA levels would be about 50% compared to controls. If there were no visible phenotype these data would most likely be interpreted as reflecting a uniform reduction in gene expression across the entire leaf. Clearly in this example complete reliance on biochemical or molecular data would be misleading, and we suggest this precise situation frequently occurs when analysing transgenic organisms.

Based on our own results and a review of the literature we show similar visible or easily scored phenotypes are remarkably common in transgenic plants; however we believe until now neither their frequent occurrence or full significance has been appreciated. In these cases transgenes behave formally as visible marker genes, thereby providing simple assays which precisely define changes in gene expression. By examining such phenotypes large populations of transformed lines can be analysed, but more importantly changes in gene expression can often be directly visualised in space (position in a plant or tissue) and time (stage of plant growth or development).

Sectored phenotypes in transgenesis

Visible sectoried phenotypes are quite common in transgenic plants. Examples from our own work are shown in Figure 2 and others from the literature (1-15) are listed in Table 1, and we are also aware of many further unpublished examples. Detailed descriptions of these plants and phenotypes can be found in the Figure legends and Table footnotes. Many of these phenotypes occur when an extra copy of a gene is expressed in either sense or antisense orientation and hence reflect sequence specific *trans* inactivation events targeting endogenous genes. However sectoried phenotypes sometimes occur when heterologous sequences such as bacterial genes are expressed in transgenic plants.

Sectoried phenotypes also occur in viral resistant transgenic plants (16-19), examples are shown in Figure 3, and others from the literature are listed in Table II. These viral resistant phenotypes manifest as alterations in the distribution of viral symptoms which for some viruses provide an indirect visual assay for viral replication (Fig. 3).

Not only are such phenotypes widespread, but often a high proportion of independently transformed lines show sectoried phenotypes (Table 1). Frequencies range from about 10% to close to 100% of transgenic lines.

The Significance of Sectoried Phenotypes

These phenotypes are significant because they occur in a variety of different circumstances, namely antisense, co-suppression and some instances of viral resistance and transgene instability. Such terms are used to describe phenomena that remain poorly understood at the molecular level, but they are commonly assumed to reflect distinct molecular processes.

In this context the occurrence of sectoried symptoms in viral resistant plants is particularly significant. The molecular processes underlying RNA-mediated viral resistance were recently brought into sharp focus by Lindbo *et al.* (20), whose results define remarkable posttranscriptional processes responsible for resistance (for recent reviews on gene silencing in plants see refs. 20-26). Their data, which will be considered in more detail below, indicate viral resistance reflects sequence-specific degradation of viral RNAs. Formal evidence links viral resistance and at least some instances of transgene instability, indicating that mRNAs from nuclear genes can also be degraded by the same mechanism.

The sectoried patterns of gene activity described above are only apparent because the events give visible or easily scored phenotypes. There is no reason to suspect that the majority of constructs, which give no obvious phenotype, will not behave in a similar fashion; however in such instances sectoried gene expression could be easily missed, since quite detailed analysis would be required before it could be detected. We propose therefore that sectoried gene activity occur much more frequently in transgenic organisms than previously imagined. Consideration of visible phenotypes in plants provides a simple means to visualise and more fully understand the full consequences of transgenesis.

Proposal: Sectoried phenotypes arise from a common mechanism

We propose that phenotypic similarities of variable sectoried patterns of gene activity, reflect common molecular events - namely activation of the posttranscriptional

homology-dependent RNA degradative system responsible for RNA-mediated viral resistance.

Initial evidence for this is based on overall phenotypic similarities that occur in transgenic plants. When considered together visible sectored phenotypes share several important characteristics. They are clearly non-uniform, regions of distinctly differing gene activity exist in sharply delineated sectors; these sectors often, perhaps always, correspond to regions of complete gene inactivation. Another striking feature is the extreme phenotypic variability that often occurs in transgenic lines; changes in gene expression occur unpredictably in space and time, generating remarkably complex phenotypes. These observations are consistent with activation of a simple binary switch which is variably induced in space and time, in what often seems an unpredictable fashion.

The available molecular evidence is consistent with this proposal and various other observations from the literature provide further indirect support. Remarkably similar events have been described in mammals and possibly other organisms, suggesting these observations have wide implications for understanding and interpreting transgenesis.

Sectored phenotypes reflect complete gene inactivation

For many of the phenotypes described above sectored regions seem to correspond to areas of complete gene inactivation, consistent with the complete degradation of mRNAs. Thus for *Flaveria* expressing PPDK sequences only background levels of enzyme activity are detected in yellow chlorotic sectors. Similarly, some phenotypes listed in Table 1, such as a complete lack of corolla pigmentation in petunia petals expressing CHS and DFR sequences or qualitative changes in starch composition in potato starch granules are also consistent with this notion. Most other phenotypes in Table 1 are fairly poorly described, but they are not inconsistent with this notion. Furthermore in instances of co-suppression where quantitative enzyme measurements have been reported, namely chitinase, β -glucanase and nitrate reductase () complete inhibition of gene activity occurs. Mechanistically viral immunity reflects complete degradation of viral RNAs, sectors of complete gene inactivation would be anticipated if they arise by a similar process.

We are aware of some exceptions to these general observations which suggest two situations where apparently intermediate phenotypes might arise. In instances where a protein or its product are relatively stable one might gain the impression of intermediate levels of gene expression. This may be the case with NADP MDH in *Flaveria* (Figure 2), where sectored regions express about 5% of control enzyme activities. NADP MDH is a chloroplast enzyme and is therefore likely to be quite stable, complete inhibition of gene activity might only be observed long after gene inactivation events occur. Another exception might arise in instances where multi-gene families are targeted for *trans* inactivation. In transgenic *Gerbera hybrida* expressing antisense CHS sequences, some lines produce pink flowers from a red parent. This phenotype is consistent with partial reductions in CHS activity (), which could reflect either partial *trans* inactivation of CHS mRNAs, or alternatively result from complete inactivation of specific members of a multi-gene CHS family that might be expressed in *Gerbera* petals. A similar phenotype has been noted in a single transgenic petunia.

Unpredictable gene expression in transgenic plants

Transgene instability in the examples shown in Figures 2 and 3 results in highly variable, unpredictable phenotypes. These must reflect complex patterns of gene inactivation which occur frequently throughout development. In some instances evidence of cell lineage relationships can be inferred, whilst in other examples gene inactivation occurs in an apparently stochastic fashion.

For example, in *Flaveria* showing unstable PPDK expression each leaf on a plant shows unique patterns of gene inactivation (Fig. 2A,B). PPDK inactivation events must initiate differently for each leaf at different stages of development. The leaf in Fig. 2C illustrates this point. The lower half is nearly fully yellow, presumably a gene inactivation event occurred early in development and involved half the leaf meristem. In the upper half gene inactivation events probably occurred much later in development, and there have been many such events. This leaf also shows evidence of a reversion event, the large green sector in the bottom of the leaf. Moreover in such plants whole shoots can be fully green, whilst adjacent shoots become completely yellow (Fig. 2E and F). Sectoring in leaves from plants expressing NADP MDH sequences also results in extremely complex essentially random phenotypes (Fig 2 G-I) which presumably reflect cognate influences on gene inactivation.

Highly variable sectoring is also seen in mini-tubers expressing PPO constructs (Fig. 2 J-L). Some tubers show only a few PPO-expressing sectors, whilst others show large areas of PPO-positive tissues. Positive sectors presumably arise from one or a few cells which either retained or regained normal levels of PPO activity during tuber formation, cones of cells presumably reflect subsequent cell division which lead to radial expansion of the tuber. Some tubers from this line showed apparently normal expression of PPO, further emphasising the unpredictable nature of phenotypes.

When alterations in viral symptoms are considered similar unpredictable phenotypic variability also occurs in PVY-resistant tobacco (Figure 4). In some lines lesion numbers on third systemically infected leaves were about 5% of controls, whilst in others numbers were 30 - 50% of controls. Lesions typically appeared to be distributed in essentially random fashion, although some degenerate patterns were observed, for example different halves of a leaf formed distinctly different numbers of lesions. In one line a highly symmetrical pattern was observed, symptoms were confined to sharply delineated rectangular regions within interveinal tissues, similar to those described by Dougherty *et al* (1994). These phenotypes also showed marked developmental influences, lesion numbers usually decreased significantly in older leaves, but even this character was not invariant, in one resistant line the number of lesions actually increased.

The pigmentation patterns described in petunia petals expressing either sense or antisense CHS sequences are also remarkably variable. Different patterns occur in individual lines ranging from highly symmetrical to apparently chaotic. Most other examples of sectoried phenotypes remain fairly poorly characterised, and have been variously described as "mottled", "chaotic" or "randomly distributed". We suggest careful consideration of these phenotypes will reveal much more information regarding phenotypic variability.

Transgene instability: current paradigms

To create transgenic plants DNA is most commonly introduced into regenerable tissues using either *Agrobacterium* or biolistics. Both approaches result in quasi-random integration of constructs. Thus in some lines constructs integrate as single copies, but often more complex patterns, such as multiple linked or unlinked integrations occur. Each individual in a population of transgenic lines therefore possesses a unique pattern of transgene integration and it is becoming increasingly clear that these markedly influence transgene activity, complex transgene integration patterns are frequently associated with aberrant expression patterns.

Position effects; cis inactivation of gene expression

The term "position effects" was coined to describe alterations in transgene activity that might reflect localised *cis* influences on gene expression. For example position effect variegation in *Drosophila* is thought to arise through localised influences of heterochromatin on gene expression, endogenous genes near blocks of heterochromatin, or transgenes which integrate near such regions, show abnormal variegated expression patterns. Similarly chance integration near strong enhancers, might influence either the total activity or developmental specificity of a particular promoter in individual lines. Similarly methylation of transgene sequences, which arises *de novo* following integration, can markedly influence gene expression, which is often thought to reflect promoter methylation leading to transcriptional silencing.

Trans inactivation of gene expression

At least two processes are known which can influence transgene expression in *trans*. Methylation patterns from transcriptionally inactive transgenes can be transferred to homologous sequences elsewhere in the genome, presumably through some type of somatic interactions between repeated sequences. Remarkably this can result in the transcriptional inactivation of unlinked loci. Although such processes have only been demonstrated for repeated transgene promoter sequences, it seems possible similar interactions could occur between duplicated coding sequences.

The second process is frequently referred to as co-suppression or posttranscriptional gene inactivation. The term co-suppression was originally coined to describe phenomena observed in transgenic petunia where attempts to overexpress key genes controlling pigment biosynthesis unexpectedly resulted in a complete block in pigment production in sectors of petals (). Expression of both the transgene and endogenous homologue were blocked in such lines, hence the term.

An extremely important shift in understanding co-suppression occurred recently with the demonstration that many instances of genetically engineered viral resistance in plants occur by this mechanism. Constructs designed to express viral structural genes in plants, such as coat protein () or polymerase genes (), often confer viral resistance. This was originally thought to result from the inappropriate expression of viral proteins which were assumed to act through poorly defined *trans* dominant effects on viral replication (). However Lindbo et al (1993) showed conclusively that the expression of non-coding viral RNAs can confer strong viral resistance, the term RNA-mediated viral resistance has been coined to describe such resistance. Such viral resistant lines show varying degrees of resistance, which manifests as either viral immunity or "recovery" from viral infection - as plants grow new tissues become viral immune; the viral resistance phenotypes shown in Figure 3 are examples of this.

Northern analysis indicated this viral immune state was associated with markedly decreased steady state transgene mRNA levels, but nuclear run-on experiments showed transcription rates remained essentially equivalent to fully susceptible tissue. These same molecular changes, namely high transcription rates associated with low steady state mRNA levels, also occur in co-suppression of nuclear genes. These results indicated that both the transgene and viral RNAs are degraded in viral resistant lines through some previously unimagined posttranscriptional process. Such viral resistance is sequence-specific, since unrelated viral RNAs are not degraded, moreover RNA degradation must occur in the cytoplasm as potyviruses, which were targeted in these experiments, replicate exclusively in this compartment.

Such data indicated that both RNA-mediated viral resistance and co-suppression are posttranscriptional phenomenon explicable only in terms of activation of an endogenous homology-based RNA degradative system. In co-suppression this RNA degradative system targets mRNAs from nuclear genes, whilst for viral resistance viral RNAs are targeted.

Antisense

In transgenic plants antisense approaches are frequently used to specifically *trans* inactivate expression of endogenous genes. The mechanism of inactivation remains unknown but antisense is widely, although not universally (), thought to differ from co-suppression. Antisense phenotypes are commonly thought to reflect duplex formation between the antisense transgene and endogenous sense mRNA, which is believed to inhibit either translation of the targeted mRNA or mark it for destruction by unknown process(es). Such models imply that antisense represses gene expression uniformly in a whole plant or tissue, which seems a widely held assumption.

Sectored phenotypes reflect posttranscriptional gene inactivation

Many of the sectored phenotypes described above reflect sequence-specific *trans* inactivation events. Processes which act only in *cis*, such as position effect variegation, cannot be responsible for these phenotypes. Moreover whilst transgene methylation can inactivate gene expression in *trans* such processes cannot possibly account for viral resistance since most plant viruses have RNA genomes.

Support for this view is based on specific molecular criteria. In instances of posttranscriptional gene inactivation, nuclear run-ons and Northern blots show that inactivated genes are transcribed at normal rates but steady state RNA levels are low (). This contrasts to transcriptional gene inactivation where run-ons show genes are not transcribed.

A review of the literature shows that at least three instances described as co-suppression () and three examples of RNA-mediated viral resistance () satisfy these molecular criteria. We are not aware of any exceptions to this, and it seems reasonable to assume that most, probably all examples of these phenomena will occur by activation of this same RNA degradative system.

Furthermore at least two instances described as transgene instability have been reported where run-ons are also consistent with posttranscriptional gene inactivation. Whilst

visible phenotypes were not reported in these examples, these results indicate transgenes can inactivate their own expression through posttranscriptional processes. Transgene instabilities that might seem to occur in *cis* sometimes reflect *trans*-acting processes. It seems reasonable to assume other examples labelled unstable expression will also occur in this fashion, especially in instances where sectorised phenotypes occur.

Less molecular data is available for antisense, we are aware of only one instance where results of nuclear run-ons have been reported. In tomatoes expressing antisense polygalacturonidase (PG) constructs, run-ons showed that transcription rates of both endogenous and antisense PG genes remain unaltered in ripening tomato fruit whilst steady state mRNA levels for both genes decreased markedly (). These results were originally interpreted using conventional models - duplexes were assumed to form between antisense and sense leading to the specific destruction of both RNAs. This interpretation is tautological, the data are equally consistent with degradation via posttranscriptional gene inactivation. We believe the latter explanation is most likely, since additional indirect evidence discussed below provides further support for this view.

Implications

Our observations suggest that posttranscriptional gene inactivation occurs very frequently in transgenic plants, which has wide implications for understanding and interpreting transgenesis.

Transgene Instability

Our observations indicate that the frequency of transgene instability has probably been grossly underestimated. This has disturbing implications.

Sectorised gene inactivation events might prove difficult to detect in instances where no phenotype occurs, especially when phenotypic variability is taken into account. Fine scale sectoring in whole tissues, or gene inactivation events in parts of plants or in transgenic progeny (either sexual or clonal) might easily be missed without detailed analysis. Furthermore environmental influences can markedly effect phenotypes. For example the frequency of co-suppression in tobacco over expressing coproporphyrinogen oxidase constructs varied markedly when plants were grown in different glasshouses, similarly plants that stably expressed a herbicide resistance gene in glasshouses showed sectorised expression in the field.

Important determinants of transgene stability can be recognised from the available literature. Complex multi-copy transgene integrations have been correlated with both posttranscriptional and transcriptional gene inactivation. Single copy transgene inserts are therefore likely to express more predictably. Furthermore to circumvent *trans* inactivation events constructs should utilise heterologous promoter and coding sequences whenever possible.

The mechanism and consequence of antisense

We have shown above that sectorised phenotypes often occur as a consequence of antisense expression. Such observations refute assumptions that antisense phenotypes

are uniform and suggests that experiments using antisense may have been frequently misinterpreted.

One prediction is that phenotypes generated by antisense and co-suppression should be identical - however for such a comparison identity must be considered in the context of phenotypic variability. In transgenic *Flaveria* expressing either sense (Figure 2H) or antisense (Figure 2I) NADP MDH constructs, very similar sectorised phenotypes occur. Similarly in PVY-resistant tobacco expression of viral sequences in either sense (Figure 4C) or antisense (Figure 4D) orientation results in the same recovery phenotype. Data summarised in Table 1 provides further support for this view, at least three other examples, CHS in petunia, ankyrin in *Arabidopsis* and probably GBSS in potato, give similar phenotypes for antisense and co-suppression.

Such a view also provides a simple explanation for the poor correlation between antisense RNA levels and phenotype which has been often noted. As described above low steady state transgene mRNA levels reflect activation of the posttranscriptional RNA degradative system. Phenotypes reflecting strong gene inactivation should therefore correlate with low transgene mRNA levels. However since phenotypes are sectorised steady state mRNA levels would be expected to vary considerably depending on the tissue or developmental stage assayed. Significantly, poor correlations between transgene mRNA levels and viral resistant phenotypes have also been frequently observed.

One difference between antisense and co-suppression is the frequency of gene inactivation events. The available data are also summarised in Table 1. In one instance (ankyrin in *Arabidopsis* and CHS in petunia) frequencies are identical, but in other examples co-suppression frequencies are higher (eg PPO in potato), markedly so for PVY resistance. The reasons for such discrepancies remain uncertain but could reflect differing propensities for activating the RNA degradative system.

The term antisense is loosely used to describe a variety of phenomena. For example in transient assays small decreases in gene expression occur when large excesses of antisense are introduced into cells. This differs from the complete gene inactivation described above; perhaps there is an effect of duplex formation, but it is small. Clearly notions of what is precisely meant by antisense require re-evaluation.

Engineering Viral Resistance

Our observations suggest the mechanism of viral resistance is sectorised viral immunity. Sectorised symptoms have been described by others in viral resistant transgenic plants (Table 2), including viruses from widely divergent genera namely potyviruses, potexviruses and tospoviruses, indicating this is a widespread phenomena. For many virus plant combinations there are particular difficulties associated with interpreting viral resistance phenotypes which might obscure similar sectorised symptoms. Thus some viral species like cucumber mosaic virus (CMV) naturally grow out of viral infections, whilst in others such as potato leafroll virus (PLRV) symptoms provide only indirect indications of viral replication. In other examples visible symptoms do not occur at all. We suggest sectorised viral immunity is probably widespread, but has often been missed.

Three common observations are frequently made for viral resistance phenotypes. Firstly when plants are challenged with virus a proportion do not develop symptoms. In those

that do symptom appearance is frequently delayed. Finally resistance often shows dose dependence, high inoculums of virus can overcome resistance. The simple model in Figure 5 can explain these observations.

There is surprisingly little evidence indicating that protein expression plays any role in conferring viral resistance, but this view has become dogma. Our observations provide a rational basis for considering viral resistance phenotypes which might help clarify such arguments.

Trans inactivation of gene expression

The ability to completely *trans*-inactivate expression of endogenous genes, or degrade viral RNAs is clearly of major practical and experimental importance. One surprising implication of our observations is that complete *trans* inactivation occurs very commonly in transgenic organisms; the available technologies of co-suppression and antisense are extremely effective.

What is required however are methods to better control gene inactivation in space and time. It seems likely that significant advances can be achieved in this respect.

Posttranscriptional gene inactivation in other taxa

It seems unlikely that a remarkable process like sequence-specific RNA degradation would be confined to plants. Many examples of distinctly non-uniform patterns of transgene expression have been observed in mammalian systems, and several key observations suggest at least some of these events occur through identical posttranscriptional processes.

In a transgenic mouse line expressing an antisense myelin basic protein (MBP) cDNA, marked decreases in both MBP mRNA and protein levels were observed. Localisation of MBP in neuronal tissues from these animals, using antibody probes, revealed MBP was distributed in a distinctly non-uniform fashion. This is consistent with sectorised *trans* inactivation of endogenous MBP expression. Furthermore in at least two examples where mammalian cell lines were transformed with antisense sequences, a poor correlation between state levels of antisense RNAs and the level of gene inactivation was noted, reminiscent of the observed situation in plants. Sectorised expression of transgenes has also been noted in transgenic mice and transformed mammalian cell lines.

Phenomena described as antisense, co-suppression or transgene instability have been described in fungal species. Moreover antisense strategies have been widely used in the study of *Dictyostelium*; and some evidence indicated that RNA degradative processes play an important role in programming differentiation processes. Such observations suggest that posttranscriptional gene inactivation is widespread.

The biological significance of posttranscriptional gene inactivation

The endogenous system responsible for homology-dependent posttranscriptional gene inactivation has remarkable properties; it seems likely such a system plays fundamentally important roles in biological systems. Others have proposed the system may normally play a role in viral resistance in plants or protect against the activity of

transposons. However the likelihood the system functions in other taxa suggests more general roles, for example one can easily envisage regulatory networks based on homology-dependent posttranscriptional processes.

In transgenic plants activation of this system frequently results in the creation of seemingly random or chaotic phenotypes, however some lines display remarkably symmetrical phenotypes. Such observations suggest that posttranscriptional processes might play an important role in pattern formation.

Our observations indicate posttranscriptional gene inactivation acts as a binary switch, rather than a "volume control" for gene expression. Intriguingly some models of enhancer action suggest they also function act as a binary switch by increasing the probability of transcription in individual cells rather than increasing transcription rates as generally believed. Assumptions that gene expression is normally fairly uniform in seemingly homogeneous tissues are perhaps overly simplistic, distinctly non-uniform patterns of gene expression may occur frequently.

EXHIBIT 5

a Construct used to create transgenic line. Plants were mostly transformed using *Agrobacterium*-based systems, except for some transgenic petunia lines which were regenerated from electroporated protoplasts. Most constructs used the constitutive 35S promoter to drive transgene expression, exceptions are for DFR, in some instances the native promoter was used and CHS, where sectorial phenotypes occur with promoterless constructs.

b Visible or easily scored phenotype which reflects transgene activity.

c Antisense refers to situations where constructs were designed to express antisense RNA sequences; Co-suppression refers to situations where sense sequences were expressed, usually full length coding sequences aimed at overexpressing a gene; transgene instability refers to situations where genes are expressed which have no endogenous homologue, such as bacterial genes.

d Frequency refers to the frequency of primary (T_0) transgenic lines displaying sectorial phenotypes.

e Transgenic *Flaveria* expressed full-length antisense *Flaveria* PPDK sequences driven by the 35S promoter.

f Transgenic *Flaveria* expressed full-length antisense *Flaveria* NADP MDH sequences or full length maize NADP MDH coding sequences designed to overexpress enzyme activity. Both constructs were driven by 35S.

g Potatoes (cv Lehmni Russet) were transformed to express antisense potato PPO sequences driven by 35S.

h Constructs designed to express either sense () or antisense () sequences driven by 35S were used to transform petunia. Sectorial phenotypes also arise when promoterless constructs are used. The phenotypes of these flowers have been particularly well characterised and show remarkable diversity (). In some instances developmental changes in phenotype (), reminiscent of the viral recovery phenotype () have been noted.

i *Arabidopsis* ankyrin encodes a *trans* regulatory protein which seems to play a central role in signalling chloroplast biogenesis. Transgenic expression of ankyrin cDNA sequences creates sectorial regions of leaf chlorosis, consistent with absence of this differentiation signal. An identical phenotype occurs regardless of whether sense or antisense sequences are driven by 35S.

j Potato tubers produce two types of starch, highly branched amylose and linear amylopectin. The two types stain differently with iodine; amylose containing starch stains blue, whilst amylose-free starch stains red. For plants expressing antisense potato GBSS sequences driven by 35S whole tubers treated with iodine stain blue in some regions and red in others, reminiscent of the antisense PPO phenotype described above. In addition individual starch grains contain blue centres and red outer regions. Since starch grains grow outwards this phenotype provides a temporal record of gene

expression in individual tuber cells. Inactivation of endogenous GBSS expression seems to be triggered early in tuber development, before starch grains are fully developed but after GBSS expression is induced. Some grains show blue concentric circles, consistent with multiple gene inactivation and reactivation events occurring over time.

k Constructs expressing sense DFR sequences and intact DFR genomic sequences, driven by the native DFR promoter, show sectorized regions of unpigmented tissues, similar to those described for CHS.

l Tobacco transformed to express tobacco nitrate reductase sequences driven by 35S show a sectorized leaf necrosis. This visible phenotype has not been extensively described, but extensive biochemical data suggests this phenotype shows developmental alterations, reminiscent of viral recovery phenotype. Quantitative enzyme measurements indicate complete gene inactivation occurs in mature tissue.

m Tobacco were transformed with constructs designed to constitutively overexpress tobacco SAM synthetase using 35S. Two phenotypes resulted; overexpression leads to leaf necrosis, but this was unstable and reverted to normal phenotype. Qualitative ? This phenotype also showed developmental influences.

n Tobacco were transformed to express coproporphyrinogen oxidase sequences driven by 35S. Overexpression was associated with leaf necrosis, which were described as occurring in "chaotic patterns".

o Tobacco were transformed to express yeast invertase sequences driven by 35S. "Chaotic patterns of necrosis" were reported which correspond to regions where invertase was expressed. No invertase activity was detectable in normal tissues?

p *Arabidopsis* were transformed to express bacterial *RoIB* sequences driven by 35S. Constitutive expression of *RoIB* generates an auxin hyper-sensitive response, leading to the creation of a severely stunted phenotype. Occasionally normal shoots develop from these plants, analysis of gene expression in these shoots indicates *RoIB* is not expressed, and nuclear run-ons indicate this inactivation is posttranscriptional. Mutants of these transgenic lines which showed a higher frequency of normal shoots have been isolated, providing formal evidence that posttranscriptional gene inactivation involves a host system.

q Transgenic tobacco resistant to the herbicide sulfonylurea were created using 35S to drive expression of the bacterial *csrI-A* gene. This confers herbicide resistance by.... In field trials of herbicide resistant plants, sectors of sensitive tissue were described as "mottled, sectorized leaves and whole plants".

Table 2: Sectored symptoms in viral resistance.

Virus ^a	Resistance Constructs ^b	Reference
Potato virus Y (PVY) ^c	PVY NIa	This paper
Tamarillo mosaic potyvirus (TaMV) ^c	TaMV coat protein	This paper
Tobacco etch potyvirus (TEV) ^d	TEV coat protein	
Peanut Stripe potyvirus (PStV) ^e	PStV coat protein	
Potato virus X (PVX) ^f	PVX polymerase	
Tomato spotted wilt virus (ToSW) ^g	ToSW N protein	

^a Refers to the viral species for which resistance was monitored. These included four potyviruses, PVY, TaMV, TEV and PStV; a potexvirus (PVX) and a tospovirus (ToSW).

^b Viral resistance in these instances is RNA-mediated. All constructs were driven by the 35S promoter. For PVY a portion of the NIa protease gene was expressed; for TaMV For TEV a non-translatable coat protein sequence; for PStV translatable and non-translatable coat protein sequences; for PVX sequences derived from the replicase gene (these were translatable but resistant was subsequently shown to be RNA-mediated) for ToSW translatable sequences from the N replicase gene which confer resistance through RNA-mediated mechanism.

^c Symptoms shown in Figure 2.

^d Symptoms described as "distinctly localised in chlorotic interveinal regions".

^e Symptoms described as "symptomatic and asymptomatic areas" where "virus was detected only in areas where symptoms were visually apparent".

^f Symptoms described as "ameliorated symptoms, characterised by isolated chlorotic lesions rather than confluent mosaic".

^g Symptoms described as ".....".

Figure Legends

Figure 1. Sectorized gene expression in transgenic plants. A single leaf of a transgenic *Flaveria bidentis* showing sectorized inactivation of endogenous PPDK gene is shown. This plant was transformed with a construct expressing antisense *Flaveria* PPDK sequences driven by the CaMV 35S promoter. In the green regions of the leaf PPDK activity is normal, whilst in the yellow levels measurable PPDK activity is only 2% of controls. In the yellow sectors expression of the endogenous PPDK gene has been *trans* inactivated and these regions are consequently non-photosynthetic. Fifteen lines were transformed with this construct, twelve were completely yellow and three gave sectorized phenotypes, further examples of which are shown in Figure 2.

Figure 2. Highly variable phenotypes resulting from sectorized gene inactivation in transgenic plants. (A-F) Sectorized inactivation of PPDK expression in *Flaveria*. (A,B) Single shoots from transgenic plants similar to those shown in Figure 1, note each leaf shows a unique pattern of PPDK inactivation. (C,D) Single leaves from such plants showing complex patterns of gene inactivation. Completely green (E) and yellow (F) shoots of *Flaveria* which grew on the same plant. (G-I) Transgenic *Flaveria* expressing NADP MDH sequences driven by the 35S promoter. The pale yellow sectors result from photosynthetic quenching which reflects the low (5%) levels of NADP MDH expressed in these sectors. NADP-MDH is expressed at about 50% of control values in green areas of leaves in these plants. (G) Plants were transformed with a construct designed to over express maize NADP MDH activity, only gene inactivation events were detected in such lines. Note each leaf shows a unique pattern of gene inactivation. (H) Single leaf from a plant expressing the sense construct in G. (I) Single leaf from a plant expressing a *Flaveria* NADP MDH antisense cDNA driven by the 35S promoter; the phenotype is indistinguishable from that in H. (J-L) Potato minitubers showing sectorized inactivation of PPO expression. These plants (cv. Lehmni Russet) were transformed to express antisense sequences from potato PPO cDNAs driven by 35S. Tubers were cut and exposed to air overnight as a crude indicator of *in situ* PPO activity. (J) The tuber on the left in was grown from a non-transformed control and turns uniformly black, the tuber on the right was grown from a transformed line. (K) Closer view of the transgenic tuber in (J), note cones of black and white tissues reflecting changes in gene activity. (L) Four tubers from the same line, note each shows unique patterns of gene inactivation.

Figure 3. Sectorized symptom formation in viral resistant plants. Viral infection in plants often results in the reproducible development of characteristic disease symptoms. For a particular combination of plant and virus, particular types of lesions (e.g. chlorotic or necrotic spots or lesions on leaves) occur reproducibly during development of the disease. For some viruses disease symptoms occur only in those tissues supporting viral replication, alterations in the distribution or appearance of such symptoms in transgenic viral resistant plants therefore provide indirect visual assays for transgene activity. (A) Non-transformed *Nicotiana tabacum* (W38) infected with PVY, leaves which form following viral challenge are invariably systemically infected and develop characteristic chlorotic lesions. In our hands this phenotype is easily scored from the third to the seventh systemically infected leaf, where the lesions are distributed quite uniformly over the entire leaf. After this point leaves are smaller and symptoms less obvious, necessitating the use of ELISA assays to monitor viral replication. (B) Symptoms in transgenic tobacco expressing non-coding RNAs derived from a portion of the PVY NIa cistron driven by the 35S promoter. Note sectorized distribution of lesions, viral particles

(detected by ELISA assays) are found only in symptomatic areas, no virus was detected in asymptomatic regions. (C,D) Systemically infected leaves from plants expressing sense (C) or antisense (D) Nla sequences relative to the PVY genome. Virus is detectable only in areas where single lesions or small foci of lesions are apparent, the same phenotype occurs regardless of orientation. These plants display the recovery phenotype first described by Linbo *et al* (). (E,F) Symptom development in *Nicotiana benthamiana* resistant to TaMV. etc

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Gerbera Paper

Position effect verigation review

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Co-suppression in mammals

Background

Recent advances in plant molecular biology have given important insights into mechanisms of *trans*-inactivation of gene expression in transgenic plants. By expressing RNA sequences driven by strong constitutive promoters transgenic plants can be created where endogenous genes can be targeted for complete inactivation or rendered viral immune. The ability to create similar phenotypes in animals has enormous implications for medicine and agriculture. This programme aims to transfer these emerging principles of gene inactivation in plants to mammalian systems.

In plants the term co-suppression refers to the inactivation of gene expression which sometimes occurs when extra copies of endogenous genes are expressed in a sense orientation in transgenic plants (for recent reviews see Meyer, 1995; Schell, 1996). Important new insights into co-suppression form the basis of this proposal. It has been known for some time that viral resistance can be created in transgenic plants can be created by expressing viral RNA genes; this resistance was initially thought to result from the expression of viral proteins, however more recent evidence indicates the mechanism is identical to co-suppression (Linbo *et al.*, 1993; English *et al.*, 1996). Furthermore it is becoming increasingly clear that antisense occurs by this same mechanism (Dougherty and Parks, 1995; Graham *et al.*, submitted for publication).

Mechanistically co-suppression involves sequence-specific degradation of RNAs, either viral RNAs or mRNAs from nuclear genes. Following infection viral resistant transgenic plants "recover" from viral infection, recovered tissues become immune to further infection. In viral immune tissues Northern blots and nuclear run-on experiments show that transgene RNAs virtually disappear even though their transcription rates remain nearly equivalent to those in viral susceptible tissues (Linbo *et al.*, 1993). Identical molecular changes, namely markedly decreased steady state mRNA levels but essentially unaltered transcription rates occur in co-suppression (Brusslan *et al.*, 1993; van Blokland *et al.*, 1994 and de Carvalho *et al.*, 1995) and antisense (Sheehy *et al.*, 1988) in plants. Since co-suppression results in the sequence-specific inactivation of RNAs, the mechanism of viral immunity must involve sequence-specific destruction of viral RNA (nearly all plant viruses possess RNA genomes). Moreover co-suppression must be a cytoplasmic phenomenon since viruses that replicate exclusively in this compartment can be targeted by this process. Furthermore this RNA degradation is sequence-specific since non-related viruses or nuclear genes are not degraded by the process. Such observations indicate the existence in plants of a previously unknown host system capable of quantitatively degrading RNAs in a sequence-specific manner.

One striking characteristic of co-suppression is that remarkably complex phenotypes arise in whole plants (M. Graham; submitted for publication). Co-suppression and viral resistance manifest as unstable sectorised phenotypes, regions of essentially normal gene activity or viral susceptibility occur in some tissues, whilst complete gene inactivation or viral immunity occurs in adjacent cells. We believe such unanticipated behaviour has led to much confusion about the behaviour of co-suppression and as described places important constraints on experimental design.

Whilst co-suppression has not been formally demonstrated to occur in mammals several key observations suggest its existence. In at least three examples where mammalian cell lines were genes have been inactivated using antisense approaches, a poor correlation between steady state levels of antisense RNAs and the level of gene inactivation was noted (Moroni *et al.*, 1992; Kook *et al.*, 1994 and Thomson *et al.*, 1995). This is reminiscent of the degradation of transgene RNAs associated with co-suppression and antisense in plants. In a transgenic mouse line expressing an antisense myelin basic protein (MBP) cDNA, marked decreases in both MBP mRNA and protein levels were observed, localisation of MBP in neuronal tissues from these animals, using antibody

probes revealed MBP was distributed in a distinctly non-uniform fashion (Katsuki *et al.*, 1988). This is consistent with sectorial *trans* inactivation of endogenous MBP expression through co-suppression. Furthermore inhibition of marker gene expression by sense constructs has been observed in transient assays in mammalian cells (Cameron and Jennings, 1991).

The aims of this programme are:

1. To establish whether co-suppression occurs in mammalian systems with an aim to obtaining dominant positions with intellectual property.
2. To define approaches to manipulate the process *in vivo* with an aim to establishing techniques to create cell lines or whole animals which are viral immune or display complete *trans* inactivation of targeted sequences.

Opportunities and Outcomes

We believe manipulation of co-suppression in animals offers novel strategies to enhance the potential applications of gene transfer into animals. Applications of such technologies might include:

- For whole animals, viral immune strains could be created or the expression of specific genes completely inactivated. The latter obviates the use of ES cells currently thought to be necessary to achieve this goal.
- Somatic cells, such as haematopoietic stem cells, could be rendered immune to viruses, a particularly potent approach for controlling viruses which infect blood cells.
- The expression of genes associated with various diseases, such as some cancers, could similarly be blocked.

Very short RNA sequences (14 bp) are capable of co-suppressing gene expression in plants (Brusslan and Tobin, 1995) and emerging data suggests that relatively short specific sequences are targeted by co-suppression. If sequences capable of eliciting co-suppression can be delivered ectopically, it might prove possible to develop novel nucleotide-based therapeutic agents.

- A detailed understanding of the molecular mechanism of co-suppression offers the potential to design new types of drugs.

The outcome of this programme will be generic patents covering animal and human transgenesis. Perhaps more importantly patents covering the design of novel therapeutic agents might also be developed.

Risks

We feel we possess both the intellectual and technical resources to make rapid progress with this research. Co-suppression has become a topic of major interest in plant research and whilst we are not aware of any groups extending this work to animals, the emerging interest in the area suggests such work is likely to commence. Competition is therefore a consideration.

It is possible that co-suppression occurs only in plants. Although we do not believe this is the case, if we fail to demonstrate its existence the programme will be terminated.

Research Rationale

The complex behaviour of co-suppression in plants indicate that careful experimental design will be critical for successfully detecting the process in mammals. Two important aspects which must be considered are the frequency of co-suppression and the complex sectored nature of co-suppressed phenotypes.

In plants many independent transgenic lines are frequently constructed but only a few lines show extreme co-suppressed phenotypes. The frequency of stable phenotypes varies considerably between constructs but is often quite low, of the order of one per cent. Typically for mammalian systems only a few transgenic events are analysed, we feel that success will require screening of large numbers of transformed cell lines. Mammalian tissue culture systems offer the ability to generate large numbers of transformation events, most experiments will therefore use such systems. For whole animal experiments large numbers of animals will be created, only those showing extreme phenotypes will be maintained as lines.

Another critical consideration is the unstable sectored nature of co-suppressed phenotypes. Co-suppression would be extremely to detect if biochemical or molecular markers were analysed since variable intermediate values would be anticipated from sectored gene inactivation events. For this reason we have chosen to analyse easily scored markers. Viral immunity in tissue culture offers particular advantages since it is an easily selected phenotype. In other experiments we will use easily scored visible markers which can be readily used to score unstable sectored gene inactivation events.

Research Programme

An outline of the research project and projected timescales is shown in Figure 1. There are two broad aims:

Objective 1: To establish the existence of co-suppression in mammals.

To increase the likelihood of success and establish suitable models for the later stages of the programme three independent strategies will be pursued:

1.1 Create viral immune lines by expressing viral sequences in stably transformed cell lines.

We will use lytic viruses for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. We will conduct parallel experiments using two unrelated viruses, a simple single stranded RNA virus (Sinbis alphavirus) and a complex double stranded DNA virus, Herpes Simplex Virus 1 (HSV 1). Both viruses are very well characterised and isolates, clones, cell lines and expertise with their manipulation are available to us.

Mammalian cell lines will be transformed with constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV) promoter. Sequences to be expressed will include specific constructs driving viral replicase genes and random "shotgun" libraries which will express all virus sequences.

For viral polymerase constructs large numbers (approximately 100) of transformed cell lines will be generated then infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines will be generated and screened in bulk for viral immunity.

Any lines obtained from such experiments will be used to more precisely define molecular and biochemical characteristics of co-suppression as outlined in Objective 2.

1.2 Inactivate the expression of nuclear genes using a simple visual reporter system.

To create a simple visual reporter we will stably transform cell lines with constructs consisting of two genes, one will express a *trans*-regulatory protein which will normally repress the expression of the second gene specifying a simple visual marker gene, the green fluorescent protein (GFP). To detect co-suppression we will target the repressor for inactivation, as a consequence GFP expression will be induced which can be easily assayed visually. To increase the likelihood of success we will prepare two sets of constructs using two different repressors, *lac* (Figge *et al.*, 1988) and *tet* (Shockett *et al.*, 1995). Expertise with these systems are available in house.

Cell lines will be transformed with these marker constructs. Cloned lines will be selected which show little or no background expression of GFP, but high levels of expression when induced by either IPTG (for *lac*) or the removal of tetracycline (for *tet*). Once characterised lines are established these could then be supertransformed with constructs expressing repressor sequences. Co-suppression could be simply monitored visually and co-suppressed lines purified for detailed analysis as described below.

Cell lines with such easily scored markers might also provide ideal systems for examining the effects of transient delivery of constructs as either gene cassettes, by using viral delivery systems or by direct delivery of oligonucleotide or oligoribonucleotides.

1.3 Inactivating pigment biosynthesis in transgenic mice.

To investigate co-suppression in transgenic animals we will target inactivation of pigment biosynthesis in transgenic mice. Pigment production in mice is well characterised genetically (Jackson, 1995), by targeting a single gene, tyrosinase, pigment production can be completely inhibited. This provides a simple visual assay, albinism in black mice, but more importantly sectoral gene inactivation events could be easily detected. Furthermore since melanocytes can be readily cultured from mature animals this system offers the ability to undertake molecular analysis of gene inactivation events.

Constructs using the CMV promoter driving tyrosinase cDNA constructs will be prepared and used to micro-inject mouse embryos. Gene inactivation events will be monitored visually and animals showing evidence of extreme albinism will be used to establish lines. Melanocytes will be cultured from such lines which will be subjected to detailed analyses as outlined below.

If no evidence for co-suppression is obtained from these experiments the programme will be terminated.

Objective 2: Molecular and biochemical characterisation of co-suppression in mammals.

Material developed from Objective 1 will potentially provide systems to study mammalian co-suppression and viral immunity using both transient and stable transformation of mammalian cell lines as well as whole animal systems. These resources will be used to undertake a detailed molecular and biochemical analysis of co-suppression. The aims of these experiments are to develop techniques which will allow the targeted inactivation of viral or nuclear RNAs at high frequency.

Cell lines obtained from 1.1, 1.2 and/or 1.3 will be characterised to determine molecular characteristics of co-suppression. Studies will concentrate on defining molecular characteristics

of co-suppression, purifying components involved in the sequence-specific destabilisation of RNAs and developing improved strategies to control the process.

2.1 Molecular characterisation of co-suppression.

Any sequences, including sequences isolated from shotgun strategies, will be re-tested to confirm their effectiveness. Transgene expression in co-suppressed lines will be examined using Northern blots and nuclear run-ons to determine whether gene inactivation occurs posttranscriptionally as seen in plant systems. Since multiple integrations correlate with co-suppression in plant systems. Southern blots will be used to determine any influence of transgene copy number.

In plants emerging evidence indicates that quite small sequences are targeted by co-suppression. The stability of various deletion and/or chimeric RNA sequences will be analysed in co-suppressed cell lines with a specific aim of defining precisely those sequences recognised by the RNA degradative process. The compilation of such data using a number of systems might suggest design rules for targeting particular RNAs.

2.2 Biochemical analysis of co-suppression.

A central issue with co-suppression is the basis of sequence specificity. Watson-Crick base pairing must be involved, therefore some form of nucleic acid must determine this specificity. The establishment of stable co-suppressed lines will offer the opportunity to purify those components involved in specifying the destruction of particular RNAs. An *in vitro* assay for RNA stability will be developed and used as the basis for purification.

An *in vitro* assay for sequence-specific RNA degradation will be established. Using this assay factors which confer sequence specificity and are unique to co-suppressed lines will be purified. Particular emphasis will be placed on defining any nucleic acids that might co-purify with such activities since such molecules presumably determine specificity and are therefore potential targets for manipulation.

2.3 Optimising co-suppression in mammals.

To effectively manipulate co-suppression in mammalian systems approaches that result in complete, stable gene inactivation at a high frequency must be developed.

It is anticipated that experiments 2.1 and 2.2 will provide rational approaches to specifically target sequences to achieve such an objective. Moreover ongoing experiments using viral resistance in plants indicate that novel types of constructs can be prepared which yield a higher frequency of stable phenotypes (M. Graham; unpublished data). We are currently extending this work and would anticipate that improved design rules applicable to mammalian systems will emerge from this work. Factors being examined include the use of multi gene constructs, the use of direct and inverted sequences and the design and use of RNA stabilising sequences.

Money

2 Research scientists, 2 research assistants x 3 years
The production of transgenic mice to be contracted out, \$60,000
Patenting costs.

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EXHIBIT 6

Interrupted palindrome

Continued From

4: 50

पृ: 25

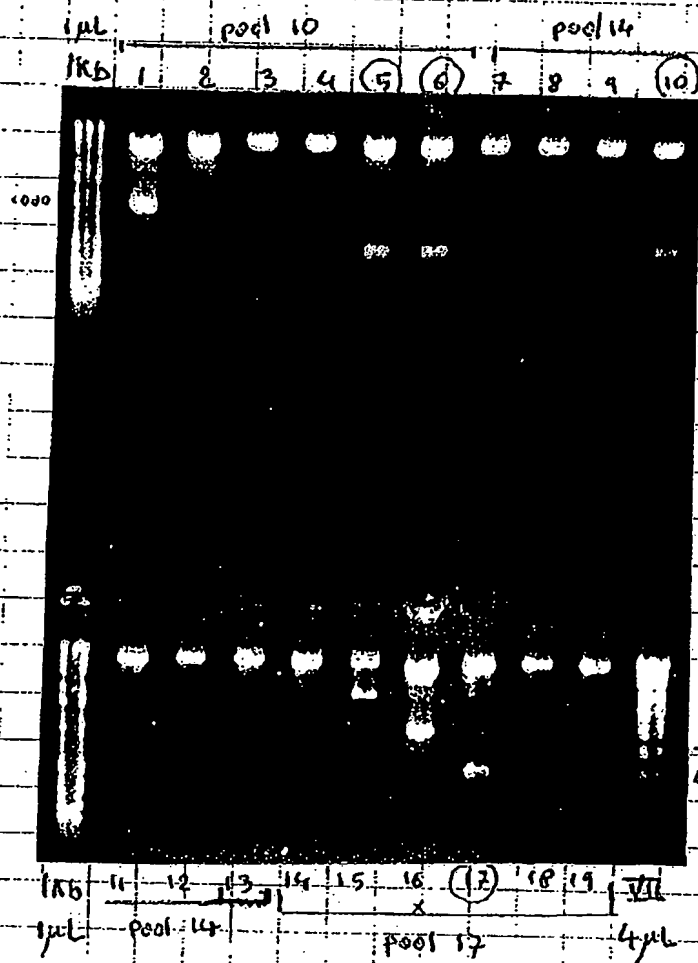
1. Miniprep of single colonies of ClapBC-PVX-LNYV + PVY (19 tubes) prepared on 11.3.98

DNA extracted, and resuspended in 100 μ L DDW. Cut for orientation of P1

Digestion:	DNA	5 μ l		stock
	buffer B	2		40 μ l
	Hind III	1	} 15 μ l of stock	20
	DDW	12		240

37°C for 1h 20min.

Gel : 8 μ L DNA digest, 94 volts. HindIII cuts.



Singles 5, 6, 10 and 17 might have the P.VY band in right orientation. Confirm with cut for both Hind III and Pst I / xba I to show presence and right orientation of the second P.VY inserted.

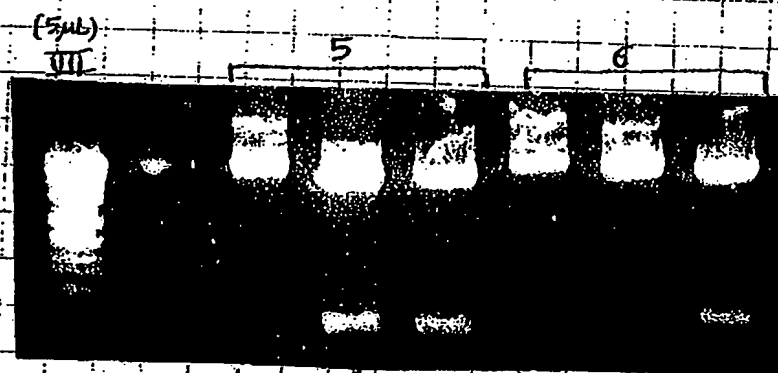
7106P
4926P

1. Digestion of *Cl*a pBC.PVY.LNYV + PVY, minis (Singles) No 5, 6, 10 and 17, both for *Hind*III cuts, and *Pst*I/*Xba*I cuts

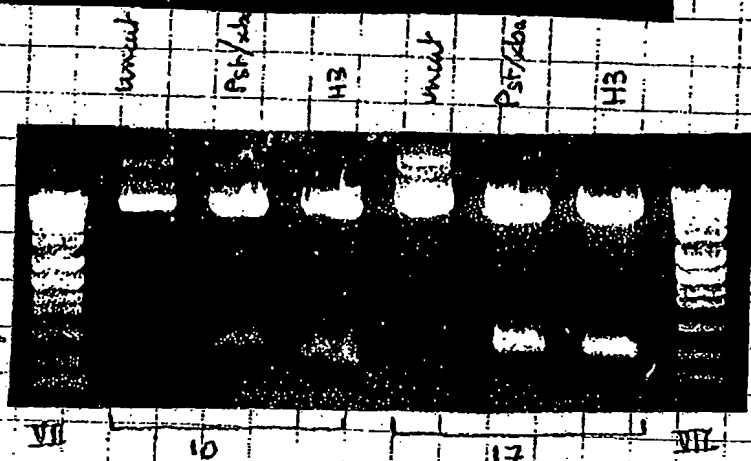
DNA	5 μ L	5 μ L
Buffer B	—	2
Buffer H	2	—
<i>Hind</i> III	—	1
<i>Pst</i> -I	1	—
<i>Xba</i> I	1	—
DDW	11	12

37°C for 3 h 20 min

Gel : loaded side by side uncut plasmid, *Pst*I/*Xba*I cut and *Hind*III cuts
uncut plasmids loaded as : 4 μ L DNA + 16 μ L DDW + 2 μ L L Buffer
digest



*Pst*I/*Xba*I fragment is smaller than the PVY band should be (750 bp) for all colonies tested



Sequencing of *Cl*a pBC.PVY.LNYV (#9)

Continued on
 # 57
 # 25

Continued From
 # 53
 # 25

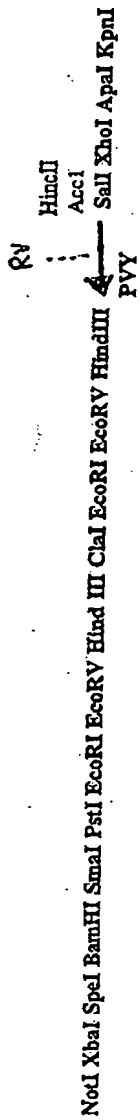
54

Title of Experiment

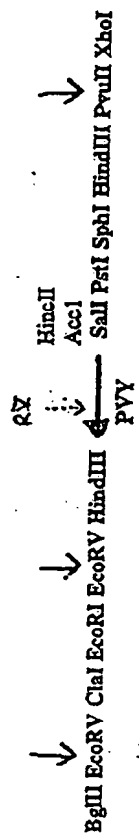
Interrupted

Reco digestion, still cutting Cla pBC.PVY.LNYV with Sma, but cutting pSP72.PVY with PvuII/EcoRV, then ligate and transform. Right still have problem with HincII/SalI/AccI sites

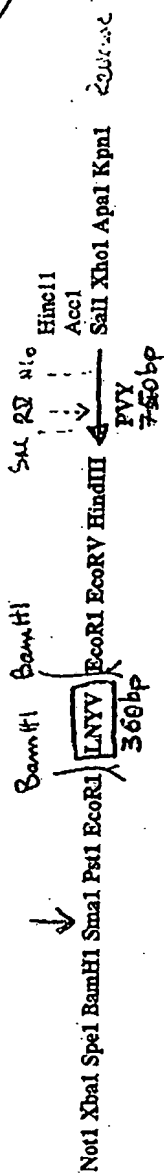
Cla pBC.PVY (ClaI/SalI pSP72.PVY into ClaI/SalI pBC.SK +)



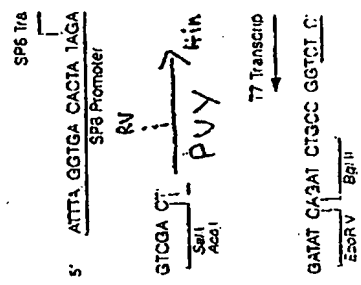
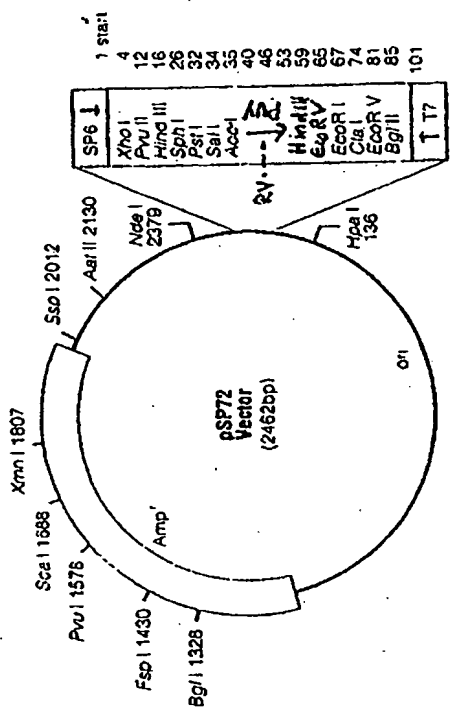
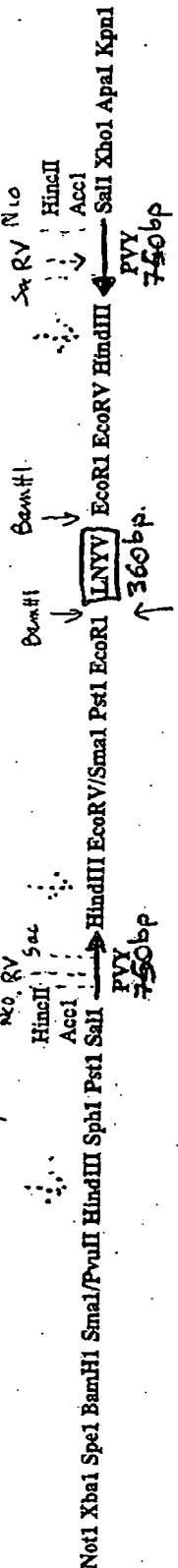
pSP72.PVY (RI/SalI pBC.PVY into RI/SalI pSP72)



Cla.pBC.PVY.LNYV (EcoRI pCR2.1.LNYV 4b into EcoRI Cla.pBC.PVY)

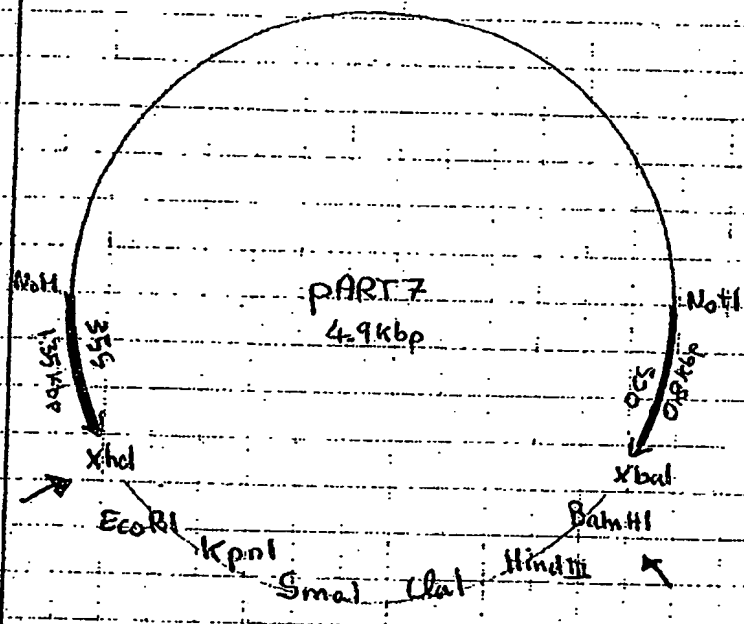


Cla.pBC.PVY.LNYV.PVY (PvuII/EcoRV pSP72 into SmaI Cla.pBC.PVY.LNYV)



3. Prepared singles for dimers pools 2 and 4
and for trimers pools 6 and 8

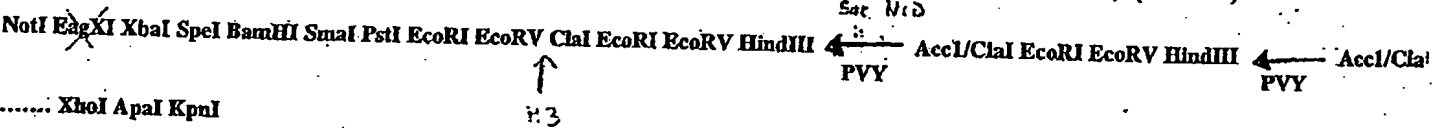
24 tubes LB with Ampicillin selection (in PART 7)



Cla pBC.PVY (AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY) (dimers)



Cla pBC.PVY (AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY) (trimers)



PVY inserts will be in this direction ← when cut with BamHI/XbaI
They will be in this direction → if cut with KpnI/XbaI

Page 54

Date

Continued From

Page #: 53

Book #: 25

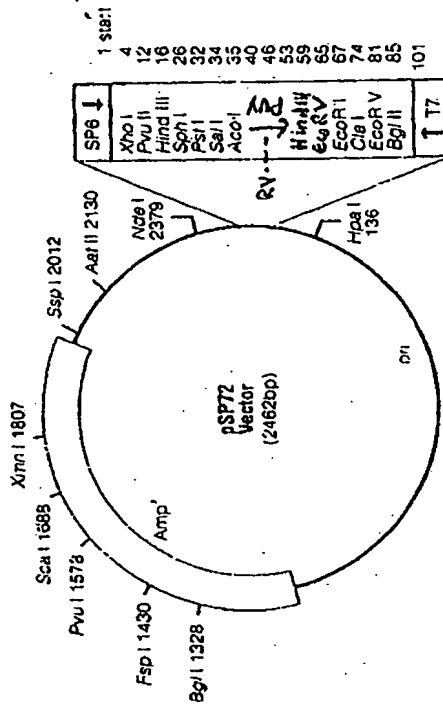
Title of Experiment

Interrupted

disc. PVY, LNYV with Sma I, but cutting PSP72-PVY with PvuII/EcoRV, then
 they had problem with HindIII/SalI/AccI site

to ClaI/SalI pBC.SK +)

RV
 HincII
 AccI
 SalI XhoI Apal KpnI
 PVY



SalI pSP72)

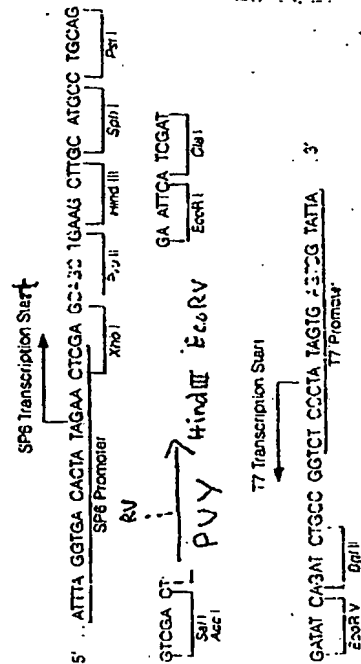
HincII
 AccI
 SalI PstI SphI HindIII PvuII XhoI

LNYV 4b into EcoRI Cla pBC.PVY)

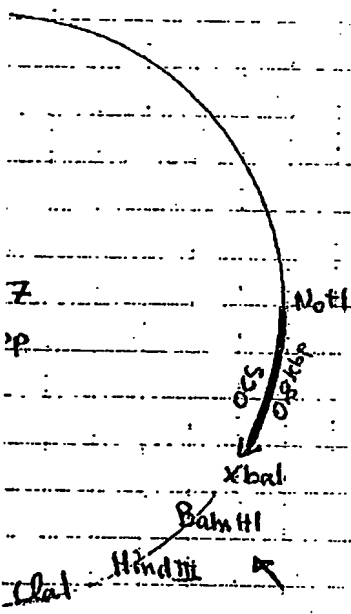
BamHI
 SalI RV N10
 HincII
 AccI
 EcoRI EcoRV HindIII
 SalI XhoI Apal KpnI
 PVY
 750bp

RV pSP72 into Sma I Cla pBC.PVY.LNYV)

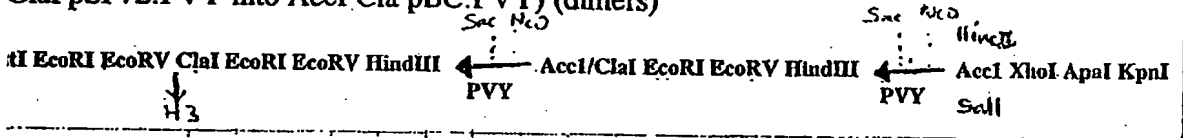
N10 RV
 HincII
 AccI
 BamHI
 EcoRI EcoRV HindIII
 SalI XhoI Apal KpnI
 PVY
 750bp
 360bp



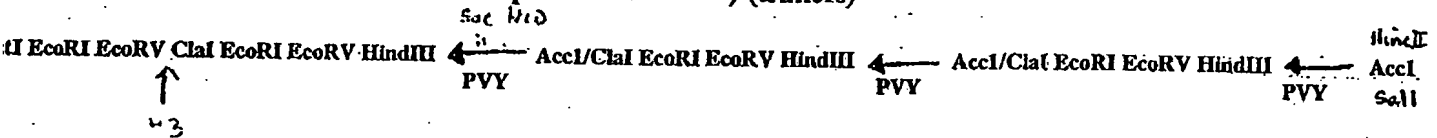
1. Ampicillin selection (in RTZ)



ClaI pSP72.PVY into AccI Cla pBC.PVY (dimers)



AccI/ClaI pSP72.PVY into AccI Cla pBC.PVY (trimers)



in this direction → when cut with BamHI/XhoI
 direction → if cut with KpnI/XbaI

Continued on
 Page #: 56
 Book #: 25

First Witness of Experiment

Second Witness of Experiment

Signed

Dated

Signed

Dated

EXHIBIT 7

Preparation of BEV constructs

Page

1

Date

6/1/98

Continued From

Page #

Book #

Overall

1) to use Bovine enterovirus as a model system to study co-suppression in mammalian cells.

2) to prepare constructs containing polymerase from BEV

3) to transfect mammalian cell line with constructs probably use Madin-Darby Bovine Kidney (MDBK) endothelial cell line

4) to challenge cells with BEV

Constructs to design

1) BEV polymerase - GFP fusion → do first (Dorelle has use pEGFP-N1 (Clontech Fusion Vector, cat # 6085-1))

2) BEV polymerase without fusion (stop codon?)

3) double promoter and terminator

not SV40

sense

antisense

4) non-translatable (not a GFP fusion)

Other mammalian promoters:

SV40

CMV

RSV

TK

Firstly need:

BEV sequence from Fiona McCarthy

↓ 4kb clone in PCR2.1 vector.

designed primers with Mike

ordered 9/12/98 from CMCB

Continued on

Page #: 2

Book #: 32

Preparing Experiment

First Witness of Experiment

Second Witness of Experiment

Signed

6/1/98

Dated

J. Hardy

6/1/98

Signed

M. Graham

6/1/98

Signed

2/2/98

Dated

1. Primer design for BEV polymerase

Date

7/1/98

Continued From

Page # 1

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Page 2

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Page 1

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Sequence Range: 1 to 1471

BEV-15CGG CAG ATG TAA CAA TGG CA

60
AGATACAGT GATACAGC GAGAGCTT TGGCTTCA TTATAGCC TATAGCCA TATAGCCA
TCTATGTA CTATAGCA CTCTGAGA ACCAATAT ACTATGCT ATATAGCT ATATAGCT
LysileProVal MetMetLeu GlycylLeu PheGlyPheLeu TyrPheVal IleAspHis>

120
GTTTACGAC CATCTGTC CAGCGCTAG CCAAGTACT TACGAGT TACGAGT TACGAGT
CAAATCGCG GTACACGAG GCTCGGAT GCTTCATTA ATCTCTTAA CCAAGTACT
SerLeuSerPro MetPhePhe HisAlaLeu AlaGluValLeu ArgAspGly GlyPheVal>

180
ATGCAACA TTCTATGAT CAGCTTCTCT GTAGCACA CCGTACAT CCGTACAT
TACGTTCT AATATAGTA GTACAGCA CAGCTGCT GTAGCTAG CCGTACAT
GluCysLysHis PheIleLeu GluLeuCys CysSerHisHis LeuTyrMet AspLysHis>

240
ATTACCTAGT CAGAGCAG CAGCTGCT GTTGGGAC TGCATCTC TGCATCTC
TATATGATA CCGTCTGAC GTACAGCA CAGCTGCT TACGAGT TACGAGT
TyrTyrValVal GlyLysMet ProSerLys CysSerGlyThr SerLeuPhe AspLysHis>

300
TAAACACT GATACAGC AGCTTACT TACTCTTA CAGACACT GATCTGAT
ATTATGCA CTATAGCA TGTATACA ACTATAGT GTATAGT GTATAGT
IleAsnLeu IleLeuArg ThrLeuVal LeuThrVal TyrAspHis AspLeuLeu>

360
ATCTAGAT TATAGCTAC GTACAGTA TACTGATC ATACGCTAT CAGTATAG
TATATCTTA AATATAGT CAGCTGCT ATACGCTAC TATAGCTA CAGTATAG
AspLeuVal IleAlaThr GlyAspLeu ValLeuLeuSer TyrPheThr GluLeuLeu>

420
CTAGCTTCT AGCGAGCC CCGAAGCT TTGCTTAT CAGACACA CCGTATAG
GATGAGCA AGCTTCTC CCGTCTGCA AGCTTCTA CAGCTGCT CAGCTGCT
AlaSerLeuLeu AlaGluLeu GlyLysSer PheGlyLeuLeu MetThrPro ProLeuLeu>

480
CCCTCAAT TGTATAGT ACTGAGCA AGCTTACT TTGAGGAG TATAGCTC
GCGTATTA AATATAGT TACGCTCT TGTATAGT TGTATAGT TGTATAGT
SerAlaGluPhe ValLysLeu ThrTyrPhe MetValThrPhe LeuLysLeu TyrPheVal>

540
CGCATAGC TTACGCTC CTGCTTAT CCGTATCA CAGTATAG CAGTATAG
CCGTAAGC AATATAGT CAGTATAG CAGTATAG CAGTATAG CAGTATAG
ArgAspThrArg TyrPhePhe LeuValHis ProValMetLeu MetSerLeu IleGluLeu>

600
CCATCTGT GAGAGAGC CCGAGCTA CTGAGACA CCGTATAG CCGTATAG
CGTATAGC CTCTCTCT CCGTATAG CAGTATAG CAGTATAG CAGTATAG
SerIleLeuThr ThrLysLeu ProArgHis ThrGluAspHis ValArgSer LeuCysLeu>

660
TGCCTGCA TGTGCTAG AAGATATA ATGATTTT TGTAGT TGTAGT
ACCGATCT AATCTGCT TGTATAT TACTTACA AATCTGCT AATCTGCT
LeuAlaThrHis CysGlyLeu LysGlyLeu AspLeuLeuVal SerLysLeu ArgSerLeu>

720
TGCCTGCA TGTGCTAG AAGATATA ATGATTTT TGTAGT TGTAGT
ACCGATCT AATCTGCT TGTATAT TACTTACA AATCTGCT AATCTGCT
LeuAlaThrHis CysGlyLeu LysGlyLeu AspLeuLeuVal SerLysLeu ArgSerLeu>

Continued on

Page # 3

Book #: 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Grahman

M. Bernard

7/1/98

V. Hardy

7/1/98

M. Grahman

2/2/98

Signed

Dated

Signed

Dated

Signed

Dated

7/1/98

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Page 3

```

1340      1360      1380
CGCTGGGCG CGCCTTCAT CTACCTCAT TTAAGGCGCT CGAAGGAGG TGTACGATT
GGCAGCGCG CGCGAACGTA GATGGAAGTA AATTCGGGA GCTTTCCTC ACCCTGCTA
ProValGlyArg AlaLeuHis LeuProSer PheLysAlaLeu GluArgLys TrpTyrAsp>

1400      1420      1440
CTTTCMAAT TCCCACTTG ATGATCCGCT TTAATTAGCT TCAATTTCGC CTGAATACAC
GAAAGCTTA ACGGTGAAC TACTAGGCCA AATTAATCGA AGTTAAACGG GACTTATGTG
SerPhe***Ile AlaAsnLeu MetIleArg PheAsn***Leu GlnPheGly LeuAsnThr>

1460
CCACCGGATG CGCTGTAAGA AAAAAAAAAA A
GCTGCTCTAC CCGACATTT TTTTTTTTT T
ProThrGlyTrp GlyValLys LysLysLys Xxxx

```

GA GGATCCCGGG

BamHI

CTCTAGG & CCC 5' BEV-2

Need to design primers with restriction sites compatible with MCS in pEGFP-N1 (see p. 4 for map)

↓
looked at restriction cut site map of BEV pol

↓
decided to use BglII and BamHI sites in pEGFP-N1 which are not present in BEV pol.

↓
when designed primers, extra sites added plus kept in frame with GFP fusion

see p. 8 for primer sequences

also note that selection of translation-initiation site sequence is from paper by Fütterer & Hohn (1994) Plant Mol Biol. 159-189

AAC AATGGC → included in primer sequence suggested most frequent AUG context of initiation region in both plant & mammalian genes

Continued on

Page #

Book #

Initiating Experiment

First Witness of Experiment

Second Witness of Experiment

Hard

V. Hardy

M. C. C. C.

Hard 7/1/98

7/1/98

2/2/98

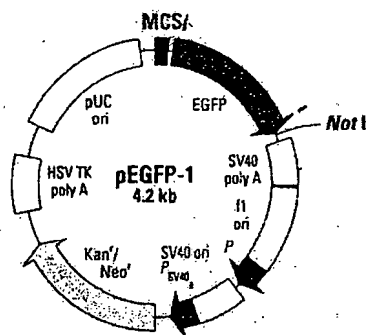
Dated

Signed

Dated

Dated

pEGFP-1 Vector



MCS

TA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG GTT
 Eco47 III Bgl II Xho I Sac I Hind III
 Ecl136 II

CBA ATT CTG CAG TCG ACC GTA CCG CGG GCC CGG GAT CC
 EcoRI PstI SalI KpnI ApaI BamHI
 AccI Asp718 I Bsp120 I XmaI SmaI
 SacII

EGFP
 A CCG GTC GCC ACC ATG GTG
 AgeI

Product	Size	Cat. #
pEGFP-1 Vector	20 µg	6085-1

Expression vector that encodes the EGFP (1, 2) variant for monitoring the activity of promoters cloned into the MCS (see the GFP introduction on pages 111–112 for further information about EGFP). Sequences flanking the EGFP gene have been converted to a Kozak consensus translation initiation signal to further increase the translation efficiency in eukaryotic cells. The vector backbone provides an SV40 origin of replication and polyadenylation sequence, and a neomycin resistance cassette for selection of stably transformed mammalian cells. An f1 origin of replication allows single-stranded DNA production, and a pUC origin and kanamycin resistance gene allow propagation and selection, respectively, in *E. coli*.

pEGFP-1 is provided with a complete vector information packet (PT3026-5) and the Living Colors User Manual (PT2040-1).

UNIQUE CLONING SITES

Eco47 III, Bgl II, Xho I, Sac I, Ecl136 II, Hind III, EcoRI, Pst I, Sal I, Acc I, Asp718 I, Kpn I, Sac II, Apa I, Bsp120 I, Xma I, Sma I, BamHI, Age I

GENBANK ACCESSION #: U55761

REFERENCES

1. Cormack, B. P. et al. (1996) *Gene* 173:33–38.
2. Yang, T. T. et al. (1996) *Nucleic Acids Res.* 24:221:4592–4593.

M. Bernard

V. Hardy

M. Graham

M. Bernard

7/1/98

V. Hardy

7/1/98

M. Graham

27/98

BEV-1 and BEV-2 primer sequences

Page

5

Date

7/1/98

Continued From

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- 394 Synthesis Setup Listing -

(Version 2.01)

Column 1

2:29:50P, 9/12/97

Run ID : BEV-1 ca 250µg
Cycle : DS 40NM
End Proc: DSCESS (DMT = Off)
Sequence: 448497B

Total bases = 38

A= 14, G= 9, C= 9, T= 6, S= 0, B= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 11705.6

START

5' > C66 CAG ATC TTA CAA TGG CAG GAG AAA TCG AGT ACA TC <3'

Egl II initiation region

Column 2

2:29:51P, 9/12/97

Run ID : BEV-2 92 µg
Cycle : DS 40NM
End Proc: DSCESS (DMT = Off)
Sequence: 448597B

purified at no extra cost.

Total bases = 31

A= 8, G= 6, C= 11, T= 6, S= 0, B= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 9420.2

5' > CCC GGG ATC CTC GAA AGA ATC GTA CCA CTT C <3'

Bam HI

Calculation of primer concentrations: (primers resuspended in 100µl sterile H₂O)

$$\text{BEV-1} \quad \frac{250 \mu\text{g}}{11705.6} / 100 \mu\text{l} = 2.14 \times 10^{-4} \text{ M} \\ = 214 \mu\text{M}$$

$$\text{BEV-2} \quad \frac{92 \mu\text{g}}{9420.2} / 100 \mu\text{l} = 9.77 \times 10^{-5} \text{ M} \\ = 97.7 \mu\text{M}$$

10µM working stocks prepared:

10µl BEV-1 @ 214µM + 204µl MQ H₂O

10µl BEV-2 @ 97.7µM + 88µl MQ H₂O

Continued on

Page #: 6

Book #: 32

Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Conrad

V. Hardy

M. Gualtani

Conrad 7/1/98

V. Hardy

7/1/98

2/2/98

Dated

Signed

Dated

Signed

Dated

PCR to amplify BEV polymerase

21/1/98

PCR reactions set up on 6.1.98

DNA template = K2577 4.2 (Qiagen miniprep DNA from Flara)
4kb clone (full sequence on file)

primers: BEV-1 and BEV-2 diluted to 1 μ M stocks

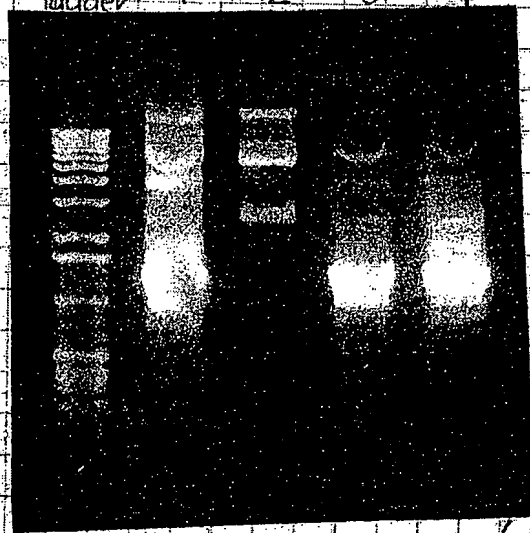
PCR con.	①	②	③ (1/10 of stock used)	④	final conc
DNA	0.5 μ l	0.5 μ l	0.05 μ l	0.05 μ l	
1.0 μ M BEV-1	2.5	2.5	2.5	2.5	0.1 μ M
BEV-2	2.5	2.5	2.5	2.5	0.1 μ M
10x buffer	2.5	2.5	2.5	2.5	
10mM dNTPs	1.0	1.0	1.0	1.0	0.4 mM
Taq BU/ μ l	0.2	0.2	0.2	0.2*	1U
H ₂ O	15.8	15.8	16.25	16.25	

old tube of Taq used for ①-③ (31 Jul 97) * new tube of Taq used (31 Jan 98)

1% TAE agarose gel:

+EBV.

500ng 1kb ladder PCR reactions (15 μ l + 2 μ l 10x loading buffer) PCR conditions 9600 PTC



Program #48 (94-47-15)

#47 94°C 0:30

60°C 0:30

72°C 1:00

#94 94°C 0:30

#15 15°C 0:30

reactions 1, 3 & 4

← 1.4kb BEV fragments for gel purification

aliquot of gel purified DNA on 1% TAE gel

← 1.4kb

Gel purification: wt of each band = 0.06g

2 bands combined = 0.12g \approx 120 μ lBresacore DNA purification kit used (360 μ l)Bresacore salt, 8 μ l Bresacore bind, DNA elutedin 20 μ l H₂O → Ligation set up 2 μ l PCR2.1 (Invitrogen) + 2 μ l insert (above) + 2 μ l ligase + 2 μ l H₂O → 14°C O/N

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bennett

V. Hardy

M. Graham

M. Bennett

21/1/98

V. Hardy

21/1/98

M. Graham

21/1/98

Signed

Dated

Signed

Dated

Signed

Dated

Preparation of DNA for cloning midipreps.

on 6.1.98, 1.0µl of each DNA transformed into 50µl DH5α competent cells using standard heat shock protocol, recovered in LB 1hour 37°C → transferred to 50ml LB + appropriate selection (100µg/ml)

- DNA (1) pEGFP-N1 kan^R
(x Darcelle Thomson)
- (2) K2S7742 Amp^R (in PCR2.1 vector 4Kb BEV clone x Fiona)

after culture grown overnight @ 37°C, shaking

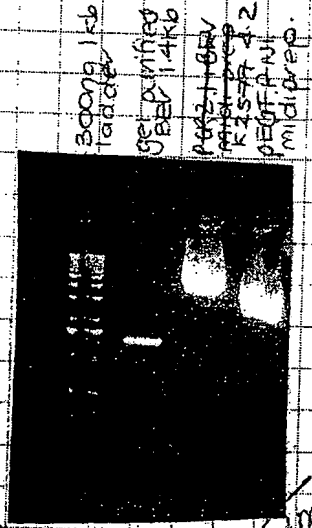
30mls culture used for BresaPure midi columns

DNA prepared as per kit protocol

DNA resuspended in 200µl sterile MQ H₂O

aliquot run on gel (2µl)

1% TAE
0.5% agarose
+ Ethidium
Bromide



7/3/98

Person Conducting Experiment First Witness of Experiment Second Witness of Experiment

M. Bernard 8/1/98 V. Hardy 8/1/98 M. Cuthbert 2/2/98
Dated Signed Dated Signed Dated

Transformation of PCR2.1 + 1.4kb BEV fragment

Date

8/1/98

Continued From

Page #: 6

Book #: 32

Invitrogen TA cloning kit used (Top 10 F cells)

3 μ l ligation from p. 6

+ Top 10 F competent cells

↓
 30' ice, 42°C 30", 5' ice, recover 1 hour @ 37°C, plated
 onto LB + ampicillin (100 μ g/ml)

↓
 37°C O/N

8/1/98 MB

8/1/98

Colony screening of BEV clones by PCR

only 10 colonies (white), 220 blue colonies.

method:

pick colony into 10 μ l LB broth, vortex, quick spin,
 add 1 μ l to PCR reaction (NB. used toothpick to pick
 colonies and also streak colony onto plate for use with
 PCR master mix minis.)

10x PCR buffer (Amgr)	2.5 μ l	x13
Mg H ₂ O	19.8	32.5 μ l
10mM dNTPs	0.5	25.4
10 μ M primer 1 (BEV-1)	0.5	6.5
2 (BEV-2)	0.5	6.5
broth	1.0	—
5U/ μ l Taq (Boehringer)	0.2	2.6
	25.0	81.2 μ l

(24 μ l / tube)

PCR reactions 1-10 (clones 1-10)

11 broth control

12 blue colony (negative control)

13 original DNA template PCR2.1 + BEV 4kb

Program 48 (changed from p. 6) now: 94 \Rightarrow 30 \Rightarrow 72 \Rightarrow 15

94°C	30"
94°C	30"
55°C	30"
72°C	1'
72°C	5'

15°C α

Continued on

Page #:

Book #:

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Graham

M. Bernard

8/1/98

V. Hardy

8/1/98

8/2/98

Signed

Dated

Signed

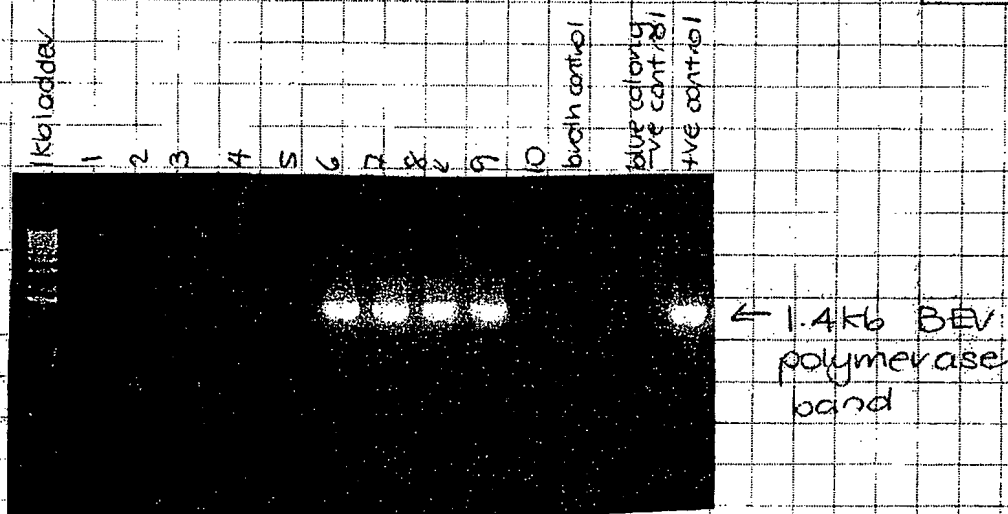
Dated

Signed

Dated

9/1/98

PCR colony screening results of PCR2.1+1.4kb BEV



clone + + + +
1 2 3 4
4 positive clones with PCR and BEV-1 and BEV-2 primers: set up miniprep cultures of clones 6, 7, 8, 9

MJB 9/1/98

3ml LB broth + kanamycin (100 µg/ml)
+ colony streak of 4 positive clones

↓
37°C O/N. MJB 11/1/98

cultures have grown ok., no time to prepare minis today. 1.5ml of each culture grown down to collect & freeze pellet. Glycerol stocks prepared

MJB 12/1/98

Continued on

Page #:

Book #:

Principal Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. Cranham

MJB

2/1/98

V. Hardy

12/1/98

MJB

2/2/98

2. Minipreps on PCR positive clones of PCR2.1+1.4kb BEV

Date

13/1/98

Continued From

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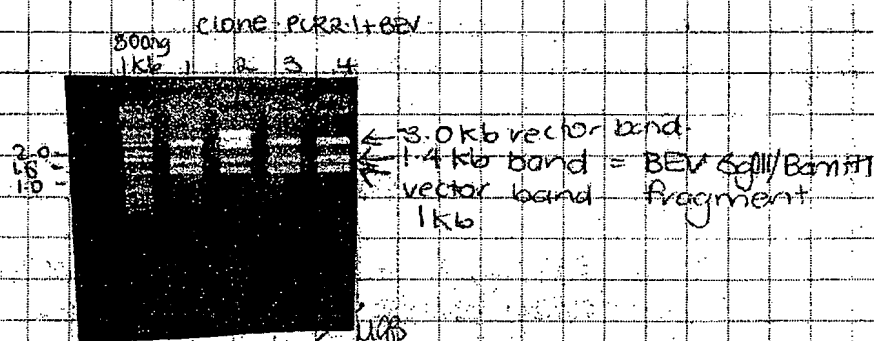
Qiagen mini spin kit used with bacterial pellets from pg. eluted in 100ul elution buffer supplied.

BglII / BamHI digests on minis

DNA	5.0ul	x4
10x buffer B	2.0	8.0
BglII	0.5	2.0
BamHI	0.5	2.0
H ₂ O	12.0	48.0
total	20.0	60.0

→ 15ul tube, added DNA, incubated 37°C 1.5hr

Total digest run on 1.5% TAE gel
90v/135', stained in ethidium bromide



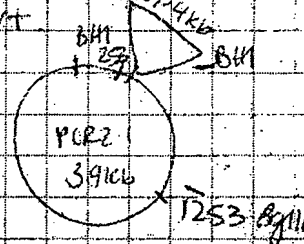
Comments:

vector PCR2.1 has a BglII and BamHI site, positions 1243 and 253 respectively... expect to see BglII another 2 bands along with the insert

PCR2.1 = 3.9 kb

insert + band (BEV) 1.4

vector bands 3.0, 1.0, 4.0 kb



Next:

select clone for further work (clone #1)
set up digests to make pEGFP-N1 + BEV

Continued on

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Book #: 32

Name of Person Conducting Experiment

M. Bernard

Signed

Dated

First Witness of Experiment

V. Hardy

Signed

Dated

Second Witness of Experiment

M. C. Graham

Signed

Dated

Cloning of 1.4kb BEV fragment into pEGFP-N1

Page

11

Date

14/1/98

Continued From

Page #:

Book #:

Aim: to digest pEGFP-N1 midiprep DNA with BamHI and BglII and use BamHI/BglII BEV polymerase as an insert.

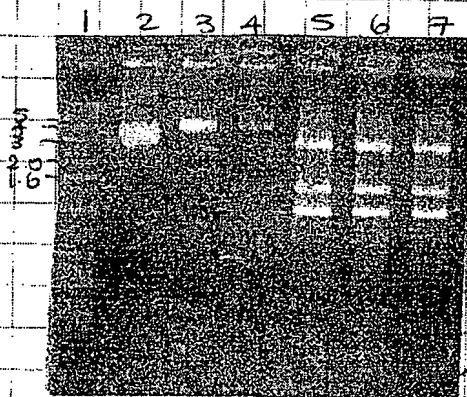
Method:

Digests

PCR2-1-BEV clone #1	15.0 μ l	pEGFP-N1 midi	6.0 μ l
10x buffer M	5.0	10x buffer M	2.5
H ₂ O	28.0	BglII	1.0
BglII	1.0	BamHI	1.0
BamHI	1.0	H ₂ O	14.5
	50.0		25.0

- incubated 37°C / 1.5 hrs, 1.5% TAE agarose gel
- total digest of BEV clone #1 run on gel over 3 lanes
- aliquot of pEGFP run on gel

- lane 1 50bp 1 Kb
- 2 2 μ l pEGFP-N1 uncut midi
- 3 2 μ l pEGFP-N1 digest → need to treat digest with phosphatase as BglII and BamHI are compatible ∴ will religate.
- 4 2 μ l PCR2-1-BEV #1 uncut mini
- 5 18 μ l PCR2-1-BEV #1 digest
- 6 " "
- 7 " "



BglII
A G A T C T
T C T A G A

BamHI
G G A T C C
C C T A G G

← 4.2kb vector band

← 1.4kb BEV bands cut out for gel purification and stored @ 4°C

Continued on

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Book #: 32

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Barnard

V. Hardy

M. Cohen

14/1/98

14/1/98

14/1/98

27/98

Cloning of 1.4kb BEV into pEGFP-N1 cont.

Date

15/1/98

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on 15/1/98

Preparation of more insert, as tube from yesterday misplaced.

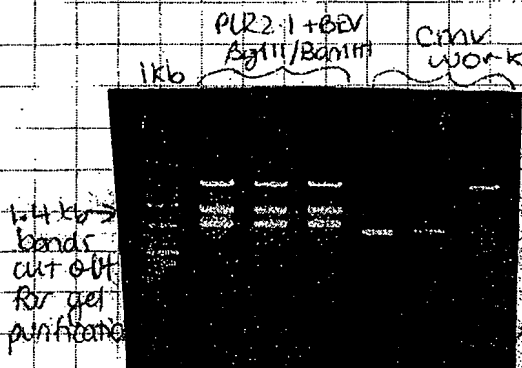
PCR2.1 + BEV #1	20.0 μ l
10x buffer M	5.0
Bgl II	1.0
Bam HI	1.0
H ₂ O	23.0
	50.0

incubated 37°C / 1.5 hours

digest run on 1.5% TAE gel 80V/35'

also found old tube!

Gel purification : see below



Phosphatase treatment of pEGFP-N1 Bgl II/Bam HI:

estimation of conc. = 0.25 μ g/ μ l
= 250 ng/ μ luse 1/2 of digest ~ 10 μ l = 2.5 μ g
2.5 μ g / 3.04 = 5.11 pmol of ends
+ 2 kb 0.01 units SAP / pmol endsShrimp Alkaline Phosphatase (Amersham) 1U/ μ l \rightarrow 1:10 dilution to 0.1U/ μ l10 μ l DNA + 5 μ l 10x Reaction Buffer
+ 1 μ l 1:10 SAP + 34 μ l H₂O = total 50 μ l
37°C / 1 hour, 65°C / 20' - aliquot run on gel

Gel purification using Bresapure DNA purification kit:

1.4kb BEV insert		
wt. of bands + gel from yesterday	= 0.25g	Amount of Bresol-salt
	= 250 μ l	750 μ l
from today	= 0.22g	
	= 220 μ l	660 μ l

6 μ l Bresol-bind used for each tubeDNA eluted in 15 μ l MQ H₂O

Aliquot run on 1.5% TAE

Gel result on p.13

Continued on

Page #: 13

Book #: 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Graham

MAB

15/1/98

Signed

V. Hardy

Signed

15/1/98

Signed

M. Graham

Signed

2/3/98

Signed

Cloning of 1.4kb BEV into pEGFP-N1 cont.

Page

13

Date

16/1/98

Continued From

Page #: 12

Book #: 32

BEV purification of pEGFP-N1 BamHI/BglII fragment

SAP treated (using BresaPure DNA purification kit)

wt. of ^{DNA solution} ~~band~~ = 50 μ l 7 μ l Bresa-bind, 150 μ l Bresa-salt
pluted in 15 μ l H₂O & aliquot was on gel.

1/ TAE gel of vector and insert aliquots

700ng 2 μ l 2 μ l
1kb PEGFP
+SAP BEV insert



1/ TAE

80V/40'

Stained in EtBr 10'

comments: smear of vector
∴ could be degradation
of vector DNA?

Ligations to set up:

	VECTOR	INSERT
(1)	pEGFP-N1 BglII/BamHI -SAP 4.2kb not gel purified.	1.4kb BEV gel purified
(2)	pEGFP-N1 BglII/BamHI +SAP	"
(3)	"	no insert

Ligations set up on 15/1/98 → O/N @ RT

	①	②	③
vector	4.5 μ l	4.5 μ l	4.0 μ l
insert	8.0	8.0	-
10x ligation buffer	1.5	1.5	1.5
Ligase	1.0	1.0	1.0
H ₂ O	-	-	8.5

Continued on

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Book #: 32

Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Harshy

M. Cuelbun

16/1/98

16/1/98

16/1/98

16/1/98

22/98

Dated

Signed

Dated

Dated

Transformations of ligations from p.13

Date

16/1/98

Continued From

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pGFP-N1/BglII + BamHI + BEV polymerase/BglII/BamHI

5ul of each ligation + 50ul DH5α competent cells only

↓
20' ice, heat shock 42°C/60", ice 5'↓
added 450ul LB broth↓
37°C 1 hour↓
plated 100ul + 200ul onto LB + kanamycin 100 μg/ml↓
37°C O/N, 4°C stored.

UAB 17/1/98

19/1/98

Transformation results: CFU (colony forming units)

1) GFP + 1.4kb BEV ⊖ SAP

plate (1) >500 CFU

(2) 7500 CFU

2) GFP + 1.4kb BEV ⊕ SAP

plate (1) ~34 CFU

(2) ~30 CFU

3) GFP ⊕ SAP no insert control

plate (1) ~40 CFU

DH5α control on LB+kan <10 CFU

Comments:

- ⊕ SAP ligation, CFU much less ∴ SAP treatment reduces efficiency of transformation as expected
- control with no insert, just vector, SAP treated shows background ∴ SAP not 100% effective.

Continued on

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Next: screen colonies by PCR

Name of Person Conducting Experiment

M. Bernard

Signed

mg3

Dated

19/1/98

First Witness of Experiment

V. Hardy

Signed

V Hardy

Dated

19/1/98

Second Witness of Experiment

M. C. Graham

Signed

M. C. Graham

2/1/98

Aim: to amplify ~1.4kb band using BEV-1 and BEV-2 primers
colonies picked → transferred to 10µl LB broth

Master mix

x 27

MS H ₂ O	19.8µl	534.6µl
10mM dNTP's	0.5	13.5
10µM BEV-1	0.5	13.5
BEV-2	0.5	13.5
10x PCR buffer	2.5	67.5
Taq	0.2	5.4
broth	1.0	—

648µl 24µl per tube

PCR reactions 1-12 = ligation 1 no run 13 or run 17
13-24 = ligation 2
25 = ligation 3
25 = PCR2.1-BEV colony #1
27 = -ve broth control

Program #92 (94→91→72→15) on 9600 (Animal lab)
annealing temp 60°C, 35 cycles, extension 1' @ 72°C

500ng 68°C/10' Finishing off 15°C 10'



no #12 when toothpick
PCR put into LB tube
misnumbering
somewhere??

<1.4kb faint bands (dubious)

Comments: ??

- positive control has
not worked (no plasmid)

- faint bands with
5, 6, 7 at 1.4kb

- stronger band with 23
- minis set up from

2.8 kb 4, 5, 6, 7, 8, 22, 24
1.4kb band and 24

Continued on

Page #: 16

Book #: 32

Bernard

V. Hardy

M. Graham

19/1/98
Dated

19/1/98
Signed

19/1/98
Dated

19/1/98
Signed

19/1/98
Dated

Date

20/1/98

Continued From

Page #: 15

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minipreps and digests on putative pEGFP + BEV clones

Clones 4, 5, 6, 7, 8, 22, 23, 24

Qiagen miniprep kit

1.5ml o/n culture

eluted in 100µl elution buffer supplied



next: digest with BglII and BamHI, which should release the 1.4kb BEV fragment

1/23/2019

21/1/98

Digests on above minis

DNA	5.0µl	x 9
10x buffer M	2.0	18.0
Bgl II	0.5	4.5
Bam HI	0.5	4.5
H ₂ O	12.0	108.0
	20.0	135.0

control

PEGFP-N1 2µl DNA
cut with BglII/BamHI

15.0µl/tube

+ 5.0µl DNA

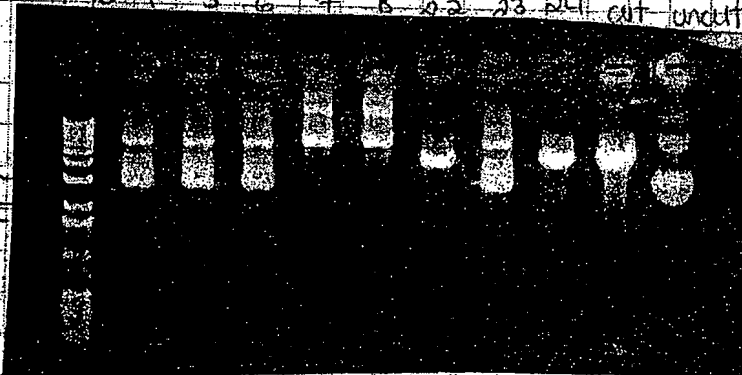
37°C 11.5 hrs, + 3µl load

1/2 of digest run on 1% agarose
gel 85V/80 mins.

700ng miniprep digests / Bgl II and Bam HI digest

1.4kb A 5 6 7 8 22 23 24 cut uncut

kbp
3.0-
2.0-
1.6-



<4.2kb vector
band

Results not as expected, no 1.4kb visible with any of the digests; ~2.8kb band (not clear) with minis 4, 5, 6 and 23, which could be a dimer of 2x1.4kb? Second band not correct size for vector, ∴ minis don't look good → set up ligation again?

Continued on

Page #: 17

Book #: 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

m. Bernard

U. Hardy

M. Graham

mbernard 21/1/98

UHardy 21/1/98

M. Graham

Signed

Dated

Signed

Dated

Signed

Dated

New ligation of pEGFP-N1 + BEV

Page

17

Date

21/1/98

Continued From

Page #: 16

Book #: 32

Aim: to set up new ligation using less vector and move insert and some new ligase

Method:

located new source of ligase (Boehringer exp 3 Jan 98)
instead of previously used stock (exp. Jun 97)

Ligations

pEGFP-N1 / BglII + BamHI + SAP (p. 13)	2 μ l
1.4 kb BEV / BglII + BamHI gel purified (p. 13)	10.0
10x ligation buffer	1.5
ligase	1.0 - 1.5
	15.0

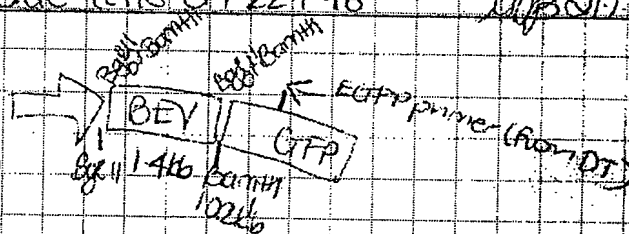
controls to check efficiency of new ligase:

	⊕ ligase	⊖ ligase
pEGFP-N1 / BglII + BamHI (p. 11, line 3)	2.0	2.0
10x ligation buffer	1.5	1.5
ligase	1.0	—
H ₂ O	10.5	11.5
	15.0	15.0

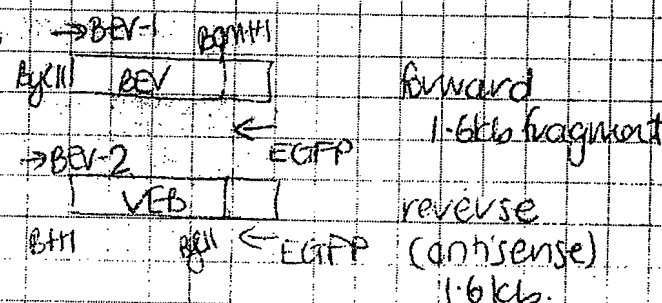
all ligations incubated ON @ 40°C
transformed into XL-1 blue cells on 22.1.98 → recovered in SOC

PCR to check ligations

use EGFP primer (from DT)
200bp downstream from
start of GFP (reverse primer)
+ BEV-1 or BEV-2



can expect 2 orientations



Reaction mix	59.4
buffer	7.5
primers	0.75
water	0.6

69 → dispensed into 11.5 μ l aliquots
+ 0.2 μ l 10 μ M primer
+ 0.5 μ l ligation reaction

Continued on

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Doing Experiment

First Witness of Experiment

Second Witness of Experiment

Signed

N. Hardy

M. Cusack

22/1/98

Dated

N. Hardy

Signed

22/1/98

Dated

M. Cusack

Signed

2/2/98

Dated

Date _____

23/1/98

Continued From

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PCR reaction

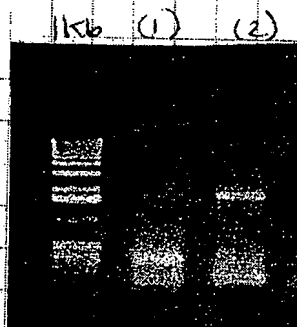
Q) Ligation of pETFP-BEV (p.17)

(2)

Primer 15

EGFP + BEV-1

EGFP + BEV-2



Result: no 1.6 kb fragment with BEV-1 primer. Forward orientation recombinants - 1.6 kb with BEV-2 primer reverse orientation or antisense recombinants in ligation.

Transformation results after O/N @ 37°C

Colonies

BAV+ pECTFP plate (1) 2 CFU / 100 μ l

(2) 2 CFM 80ml

④ house control

65x8 CFM 100ml

① House control

9 CFH/100ml

-ve controls (xl-blue) on K100 (EU 11091)

$$\Delta_{100} \leq 0.1/100 \text{ m}$$

No ligation mix left as added. $\text{Apo} \leq 0/100 \mu\text{l}$.
 as added. No. to ligh instead of w/ blue cells.

Comments:

only 4 colonies to screen for transformants

unlikely to find we close

→ something wrong with transformation? other work done in parallel & larger no.'s colonies obtained

Could plate out more transformation but may be better off starting again.

Decided to screen 4 colonies by PCR (BEV-1 and BEV-2 primers) + old ligations from 4.5.1.98

See p. 19

Continued on

Page 5.

Book 4

Name of Person Conducting Experiment:

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardin

M. C. Jones

Maßmann

224 1 1978

W. H. Anderson

23/1/98

2

PCR to check colonies + old ligation (15.1.98)

Date

25/1/98

Continued From

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Book #: 32

BEV master mix for colony screening

H ₂ O	9.9 μ l	79.2 μ l
primers	0.25	2.0
BEV-1	0.25	2.0
BEV-2	0.25	2.0
Am Taq	0.1	0.8
buffer	0.25	10.0
broth	0.5	—
	12.5	96

Program 48 #94 94°C 0.90

94°C 0:30

#47 55°C 0:30

72°C 1:00

#72 88°C 5:00 15°C α .

x30 cycles

12 μ l dispensed per tube + 0.5 μ l broth + colony

Reactions

1-4 = colonies 1-4

5 1:25774.2 0.6 μ l of 1:10 diln6 PCR 2.1 + BEV clone #1 0.6 μ l

V vector only colony from control transformation (religated vector)

B broth only (-ve control)

ligation

PEUTFP + BEV (p.13) = ligation 2

reaction

1 l'ign 2

2 "

3 control PEUTFP IN VECTOR

primers

BEV-1 + EUTFP

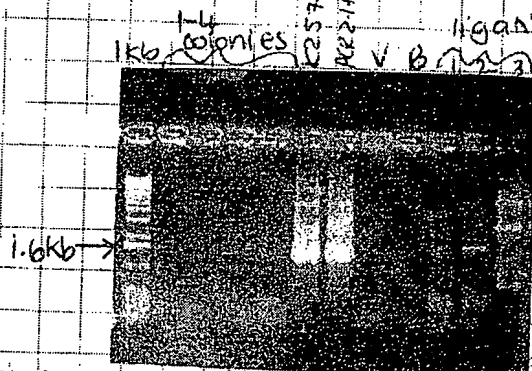
BEV-2 + EUTFP

EUTFP

can't remember which other primer used

MOD 13/98

1. TAE agarose gel



ligation results:

no 1.6 kb fragments

indicates no recombinants in

this ligation.

1.6 kb bands with PEUTFP

may have to check again.

Results: no positive clones from colonies 1-4 (didn't really expect to find one!), controls all ok. Maybe colony PCR not working well (toothpicks causing inhibition?), as positive controls are plasmid DNA. NEXT: START AGAIN.

Continued on

Page #: 20

Book #: 32

First Witness of Experiment

Second Witness of Experiment

Signed

U. Hardy

M. Graham

25/1/98

Hardy

25/1/98

Graham

27/98

Dated

Signed

Dated

Signed

Dated

26 1/98

Cloning started again. . . . PEGFP-N1 + 1.4kb BEV

Vector	PEGFP-N1	Insert	BEV from PUR2-HBEV clone #3
midiprep DNA	10 μ l	DNA (midiprep #3)	10.0 μ l
Bm10x buffer M	2.5	10x buffer M	2.5
H ₂ O	10.5	Bgl II	1.0
Bgl II	1.0	Bam HI	1.0
Bam HI	1.0	H ₂ O	10.5
	26.0		25.0

incubated 37°C / 1.5 hrs

Dephosphorylating vector (using shrimp alkaline phosphatase)
SAPto 25 μ l digest1.0 μ l SAP (10 μ l) Amersham3.0 μ l 10x SAP buffer1.0 μ l H₂O

inc. 37°C / 1 hour

heat inactivated 65°C / 15 mins \rightarrow iceTotal amount of vector + insert run on 1% agarose gel
(photo in book 8, p. 59)

vector band at 4.2 kb (still a lot of DNA in well?)

insert band at 1.4 kb

bands excised for gel purification

Gel purification (using QIAprep DNA purification kit)

vector 0.11g \approx 110 μ l 330 μ l QG bufferbrand new Eppendorf
Paul Campbellinsert 0.08g \approx 80 μ l 240 μ lboth eluted from column in 30 μ l H₂O, speedivac low / 10
aliquots run on gel. (see p. 21)

Name of Person Conducting Experiment

M. Bernard

M. Bernard

26 1/98

Dated

First Witness of Experiment

V. Hardy

V. Hardy

Signed

26 1/98

Dated

Second Witness of Experiment

M. Graham

M. Graham

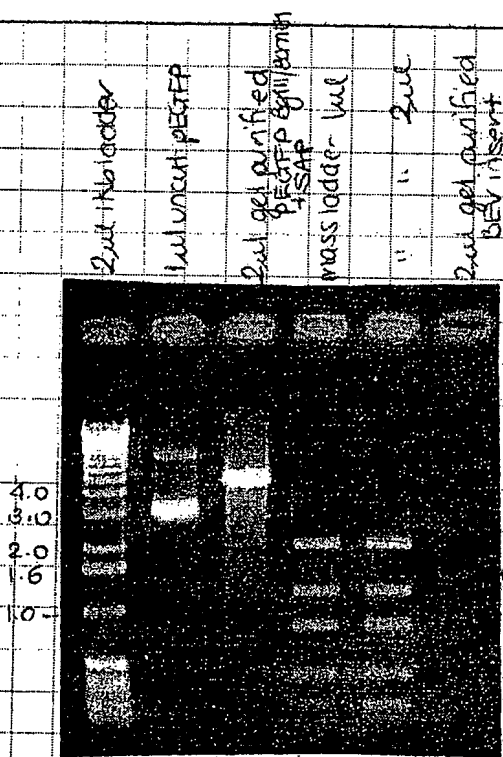
2/20

26/1/98

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Concentrations of DNA for ligations

Approx. concentrations of DNA: ^{MB} 26/08

	vector	insert	vol.	yield ng	size ratio	amt. DNA
	2100ng/2ul ~2500ng	10ng/2ul	19ul 20ul	2335 100	~3 1	100ng 100ng

ligation (+insert)

0.5ul vector
 16.5ul insert
 2.0ul 10x ligation buffer
 1.0ul ligase (3130ng)
 20.0ul

(-insert)

0.5ul vector
 1.0ul ligase
 1.5ul 10x buffer
 12.0ul H₂O

inc. O/N @ RT

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Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. Guzman

26/1/98

V. Hardy

26/1/98

M. Guzman

27/1/98

Dated

Signed

Dated

Signed

Dated

PCR to check ligations

Date

27/1/98

Continued From

Page #: 21

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Aim: to check ligations and to transform ligations into xli-blue

METHOD: PCR master mix x 6

H ₂ O	9.94 μ L	59.65
BM10x buffer	1.25	7.5
10mM dNTPs	0.21	1.25
Taq	0.40	0.60

69 \rightarrow 11.5 μ L aliquots, + 0.25 μ L each primer
+ 0.5 μ L ligation. (primers)

LIGATION	10 μ M PRIMERS
PEGFP + BEV	BEV 1 EGFP BEV 2 EGFP
PEGFP -	BEV 1 EGFP BEV 2 EGFP

Program #48

20 cycles / 55°C

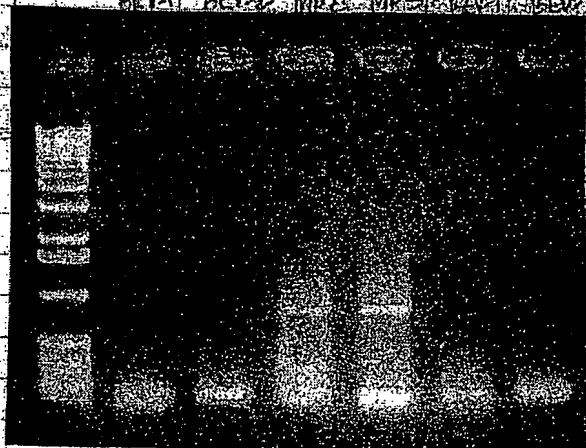
OTHER WORK

PSP42 del	MP2	T7
+cmv.mpr	MPE	T7

1. TAE agarose gel, 90V/40'

WB 27/1/98

PEGFP + BEV PSP42 + cmv
BEV 1 BEV 2 MP2 MPE PEGFP - insert
BEV 1 BEV 2



no 1.6 kb bands with any primer set for PEGFP + BEV ligation

Continued on

Page #:

Book #:

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Gauthier

Signed

28/1/98
Dated

Signed

28/1/98
Dated

Signed

2/5

Genet. Transformations into XL-1 blue cells.

Page

23

Date

28/1/98

Continued From

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cn27.1.98

competent cells - XL-1-blue (chemical)

500µl cells ⇒ dispensed in 100µl aliquots

7µl of each ligation, heat shock method,
recovered in 500µl SOC media 1 hour / 37°C

inc. 37°C O/N	selection	# colonies
PEGFP + BEV	kan	≤ 0
PEGFP (no insert)	"	≤ 0
controls		
PSP32-DE/ECORV + ligase	Amp	≤ 0
" " - ligase	"	≤ 0
XL-1-blue control	Amp	≤ 0
"	kan	≤ 0

Comments:

transformation has not worked for some reason.
competent cells could be a problem, although
a transformation for Vanessa performed at the
same time, resulting in some colonies.

What next??? Sequence PCR2.1+BEV clones.

Set up new ligation using already prepared vector
and insert.

6.0µl BEV insert (1.0µl from old preparation
on 15.1.98 and 5µl of newly
0.5µl vector (as on prepared on 26.1.98)
p.21)
1.0µl 10x buffer
1.0µl ligase (31 Jan 98)
1.5µl H₂O
10.0µl inc. on bench for 3 hours

Ligation desalted using Amicon microcon columns
(concentration 30, no. 42410) (also desalted 26.1.98 lig)
Ligation of 10µl + 480µl H₂O, 2.5K / 30 mins
in column, final volume 100µl PJO.

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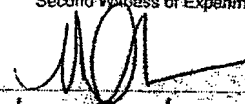
Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

David

V. Hardy



28/1/98

Dated

V. Hardy

Signed

28/1/98

Dated

M. Cavanagh

Signed

10/2/98

Dated

Expt. 5: Transformation of pEGFP+BEV ligation

Date

28/1/98

Continued From

Page # 23

Book # 32

Aim: - to transform ligation from p.23 into electrocompetent cells
 - also to check transformation protocol by using pUC18 as a control

Cells:

JSH Biorad RecA+ (from Roger Murchau) electrocompetent
 DH5α chemically competent

JSH	12.5ul	1ul	pUC18 (10ng)
JSH	12.5ul	2ul	PEGFP+BEV (26.1.98 ligh)
JSH	12.5ul	2ul	PEGFP+BEV (28.1.98 ligh)
DH5α	20ul	1ul	pUC18 (10ng)

Electroporation method:

conditions: 1.5V, 25uF, 200R, 0.1cm cuvette
 recovery in SOC, 37°C/50'

Heat shock:

42°C/60", recovery in SOC/37°C/50'

Time constants after electroporation, all ~4.5

50-100ul plated out onto selection, Inc. O/N @ 37°C

#

Continued on

Page # 25

Book # 32

Name of Person Conducting Experiment

M. Bernard

M. Bernard

28/1/98

First Witness of Experiment

V. Harary

V. Harary

28/1/98

Second Witness of Experiment

M. Graham

M. Graham

10/1/98

Results of transformations

Date

2/2/98

Continued From

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Book #: 32

from 28.1.98:

TNTC
too numerous
to count

plate

#colonies

25 μ l JS4 + pUC18 (10ng)TNTC / 80 μ l

small + large colonies

20 μ l DTF5x + pUC18 (10ng)TNTC / 80 μ l

less than with JS4

JS4 + pEGFP (26.1.98)
+ BEV ≤ 0 JS4 + pEGFP (28.1.98)
+ BEV

1

Paul plated out remainder of transformation (new
Apo plates for pUC18) 1500 plates already prepared
for pEGFP ligation.

plate

#colonies

JS4 + pEGFP^{BEV} (26.1.98) ≤ 0

JS4 + pEGFP + BEV (28.1.98)

 ≤ 0

Comments: results are not very promising. pUC18
controls gave alot of colonies (unable to work out
efficiency), but only one colony with pEGFP + BEV
ligation from 28.1.98. Need to make more comp cells.

Discussions with Mick \Rightarrow start again, don't use any
SAP or gel purification. Try counterselection method.

PCR2.1 + BEV clone #1

kan^R Amp^R

pEGFP

kan^R

\therefore choose colonies that only have kan^R.

Continued on

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Book #: 32

Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. Gresham

2/2/98

Dated

V. Hardy

Signed

2/2/98

Dated

M. Gresham

Signed

2/2/98

Dated

New digests of vector & insert.

Date

21/2/98

Continued From

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Book #: 32

Aim: to set up new ligation using no gel purification.

PCR2.1 + BEV #1

15 μ lpECTP-NI
(xDT03 μ g/ μ l)10 μ l

BM10x buffer M

5

BM10x buffer M

5

BM BglII

1

BM BglII

1

BM BamHI

1

BM BamHI

1

H₂O

28

H₂O

33

50

50

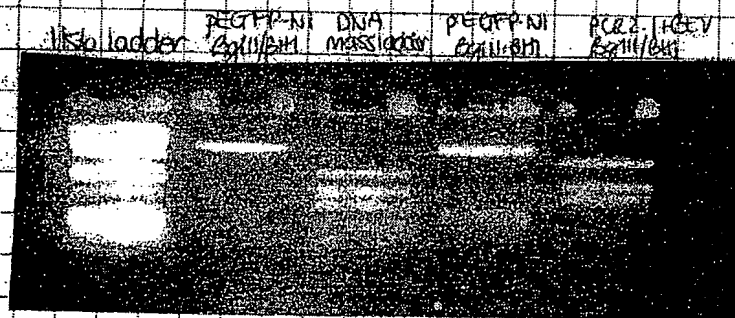
inc. 37°C 12 hours

DNA ppt'd - 125 μ l 100% EtOH + 5 μ l 3M NaOAc, pH 5.2
freezer 10', 14K 120' @ 4°C, pellet wash
in 70% EtOH, pellet dried & resuspended
in 10 μ l H₂O.

0.8% TAE agarose gel, 100V

lane

- 1 2 μ l 1kb ladder
- 2 1 μ l digest pECTP-NI BglII/BamHI (14.1.98)
- 3 1 μ l DNA mass ladder
- 4 1 μ l digest pECTP-NI BglII/BamHI (today's)
- 5 1 μ l digest PCR2.1 + BEV #1 (today's)



1 μ l DNA mass
ladder

50
20
10
5
2.5

ligations set up:

	14.1.98	2.2.98
vector	2.5 μ l	2.5 μ l
insert	4.5	4.5
ligase	1.0	1.0
H ₂ O	5.5	5.5

inc. RT O/N on bench.

Continued on

Page #:

Book #:

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Cranham

11/13
Signed21/2/98
DatedM. Bernard
Signed21/2/98
DatedM. Cranham
Signed10/2/98
Dated

Aim: to transform 2 ligations from p.26 into DH5α chemically competent cells prepared yesterday.

Method:

100µl DH5α

① ligation 2 (14.1.98) 4µl

② ligation 2 (22.98) 4µl

③ 1pg pUC18

1µl of 1pg/µl dilution (used 1µl of pUC18 stock 10ng/µl to prepare dilution)

④ 50pg pUC18

1µl of 50pg/µl dilution

-ve control

ice, 20-30', heat shock 42°C/90", recovery in 900µl SOC
plated out 100-200µl onto kan 100 µg/ml or
amp 100 µg/ml plates.

inc. O/N @ 37°C

Continued on

Page #:

Book #:

Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Gernard

V. Hardy

M. Graham

4/2/98
Dated

4/2/98
Signed

4/2/98
Dated

4/2/98
Signed

4/2/98
Dated

Transformation results:

Date

5/2/98

Continued From

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to calculate transformation efficiency to new
prepared DH5α cells:

370 colonies / 100 μl plated out from 50 pg
transformation

$$= 5 \mu\text{g DNA} \times 200 = \text{ng DNA}$$

$$\times 1000 = \mu\text{g DNA}$$

$$74 \times 10^6 \text{ CFU} / \mu\text{g DNA}$$

$$= 7.4 \times 10^7 \text{ CFU} / \mu\text{g DNA}$$

Reasonable efficiency with new cells. Checked on
kan and amp plates for resistance, no colonies
observed after O/N inc @ 37°C.

Result of transformation with ligation ① & ②
= pECTFP-N1 + BEV.

Lign ① 100 μl + 200 μl plates
colonies TNTC

colonies selected for counterselection
on both kan + amp plates.

② same as above.

Expect alot of religated vectors.

either PCR2.1 / BglII + BamHI

amp^R / kan^R

pECTFP-N1 / BglII + BamHI

kan^R

2 both plates
each ligat

on counterselection plates looking for kan^R or
colonies, as there should be two pECTFP-N1 vectors

Continued on

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Name of Person Conducting Experiment

M. Bernard

Signed

Dated

First Witness of Experiment

V. Hardy

Signed

Dated

Second Witness of Experiment

M. Graham

Signed

Dated

Counterselection results + PCR screening of putative transformants

Date

6/2/98

Continued From

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counterselection results

37 Kan^R colonies from ligation 166 Kan^R colonies from ligation 2

PCR screening:

Method: one colony per 10 μ l LB, toothpick shaken in LB briefly & discarded. LB tubes placed at 37°C while setting up PCR master mix. Vortex & briefly spin tubes before adding both BEV master mix to master mix

		$\times 25$
H ₂ O	9.9 μ l	247.5 μ l
10mM dNTPs	0.25	6.25
10 μ M BEV-1	0.25	6.25
BEV-2	0.25	6.25
10 \times PCR buffer	1.25	31.25
BM Taq 5 μ l	0.10	2.5
broth	0.50	-
	12.5	300.0 \rightarrow 12 μ l per tube + 0.5 μ l broth 1 colony

Program #48

94 \rightarrow 47 \rightarrow 72 \rightarrow 19

#47

94°C 30" } $\times 35$ cycles
 60°C 1' }
 72°C 1' }
 68°C 5'
 15°C ∞

gel loading buffer added to samples and run on 1% TAE agarose gel. 90V/35".

Continued on

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Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

J. Hardy

M. Graham

6/2/98

6/2/98

6/2/98

Dated

Signed

Dated

Dated

6/2/98

1-22

colonies 1-22

23

kan^R Amp^R colony

24

broth control

25

PCR2-1+BEV #1 0.5 μ l plasmid DNA

lane

1

0.4 μ g / kb

2

1

3

2

4

3

5

4

6

5

7

6

8

7

9

8

10

9

11

10

12

11

13

12

14

1/2 of 25

faint bands with runs 1, 3, 8, 10

1

0.4 μ g / kb

2

13

3

14

4

15

5

16

6

17

7

18

8

19

9

20

10

21

11

22

12

23

13

24

14

1/2 of 25

faint bands with runs 17, 18, 20, 22

strong band with run 19

run 23 kan^R Amp^R colony could be PCR2-1+BEV

24 -ve broth control ok

25 -ve control ok

Name of Person Conducting Experiment

M. Bernard

mqb

Control

6/2/98

Dated

First Witness of Experiment

V. Hardy

V. Hardy

Signed

Second Witness of Experiment

M. Graham

6/2/98

Dated

Minipreps of putative transformants.

Date

8/2/98

Continued From

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LB+kan 100 µg/ml cultures set up o/n @ 37°C.
pEGFP+BEV

singles: #1, 3, 8, 10, 17, 18, 19

pools: kan^R colonies selected

ligation	colony # on plate
1	23, 24, 25, 26, 27, 28, 36
2	29, 30, 31, 32, 33, 34, 35

ligation	pool #	colonies
1	1-3	4 + 3 colonies
2		8 colonies
4	19	4 + 3 colonies
5		6 "
6		6 "
7	+ 14	4 + 3 "
8		4 "
10		4 "
11		5 "
12		3 "
13		5 "
16		5 "

grown ~23 hours @ 37°C.

UB 8/2/98

Qiagen minipreps, eluted in 100 µl, digested 5-15 min with BamHI / BglII. Some cultures did not grow.

BEV pools

		x14
DNA	15.0	
10x buffer M	2.0	28
BglII	0.5	7
BamHI	0.5	7
H ₂ O	2.0	28
	20.0	70

→ dispensed into
5 µl aliquots

BEV singles

		x4
DNA	5.0	
10x buffer M	2.0	8.0
BglII	0.5	2.0
BamHI	0.5	2.0
H ₂ O	12.0	48.0
	60.0	

→ dispensed into
15 µl aliquots

DNA added, inc. 2 hours @ 37°C.

PTO gel results of digests

Continued on

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M. Bernard

mjb

9/2/98

Dated

V. Hardy

V Hardy

Signed

9/2/98

Dated

M. Graham

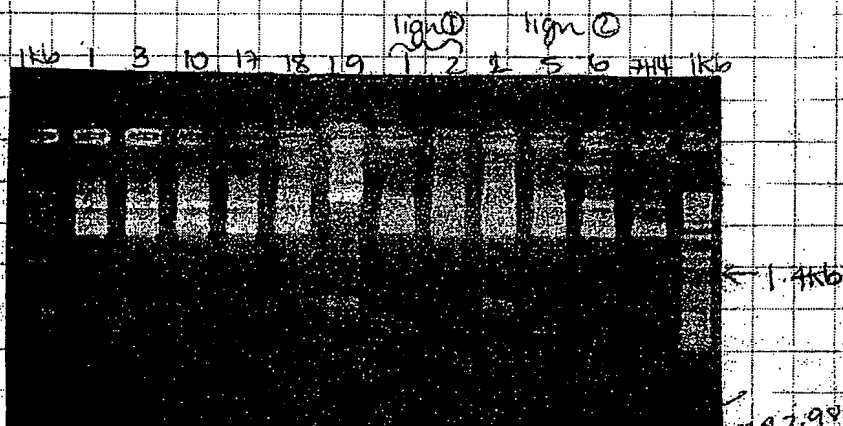
M Graham

10/2/98

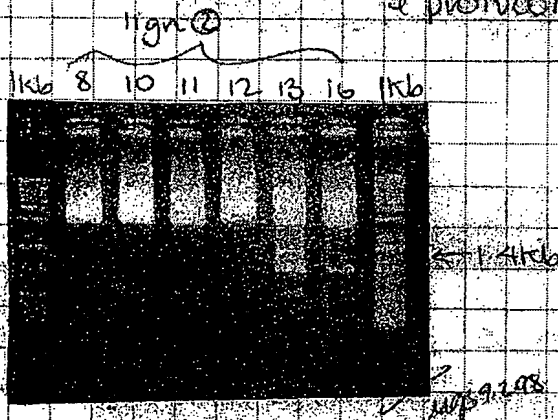
Dated

9/2/98

Digests results on minipreps:



NB note with most of minis (except 3u16 from lign 2) were spun down before adding buffer NB, resuspended & 650ul NB added & protocol cont.



Results: - no 1.4Kb bands in top gel. Expected a band with #19 as there was a strong band with PCR (#19 could have been a Kan^R Amp^R colony?)
- 1.4Kb with pool #13 from ligation 2.

Next: Set up singles from pool #13
+ repeat #19

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Book #:

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Graham

M.B.

9/2/98

V. Hardy

9/2/98

M. Graham

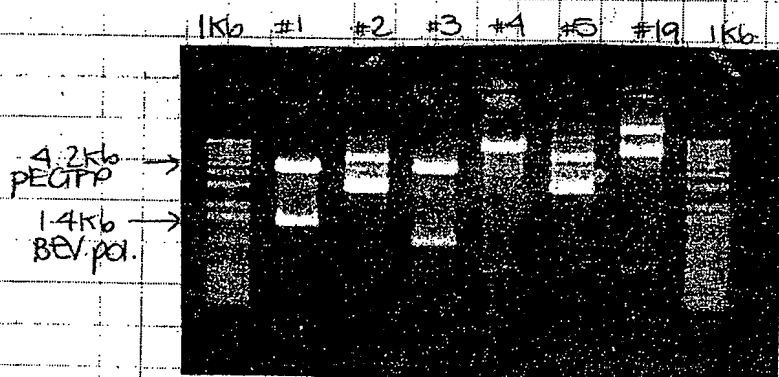
Aim: to check single minipreps from pool 13 (p. 32)
and to repeat clone #19 (p. 30)

Method: ①-⑤ = pool 13 (5 singles) ⑥ = repeat of #19
Qiagen miniprep spin columns on 2ml O/N cultures
eluted in 100µl nuc. H₂O
digested Sul DNA with BglII + BamHI

	DNA	5:0	x 6
	BM 10X buffer (M)	2.0	12.0
	BM BglII	0.5	3.0
	" BamHI	0.5	3.0
	H ₂ O	12.0	72.0
		20.0	90.0

Sul aliquot
+ DNA, inc. 37°C / 1.5 hrs.

RESULTS:



1% TAE gel
80V / 45'
stained in EBEV

- only one clone has a 1.4Kb fragment, expected to be the BEV polymerase. → clone #1 from pool 3

NEXT:

- sequence clone #1 → check which primers to use.

V. Hardy

10/2/98

Dated

V. Hardy

Signed

10/2/98

Dated

M. G. G. G.

10/2/98

Dated

11/2/98

Sequencing of pEGFP-N1 + BEV polymerase done
plus PCR2.1 clones + BEV. pol.

PCR2.1 + BEV polymerase

(sequencing reactions set up on 28.1.98 using original
FS mix)

		Reaction	Primer
8.0 μ l	FS mix		
0.3 μ l	primer	1 PCR2.1 + BEV clone #1	Univ. 1
5.0 μ l	miniprep DNA	2 "	Univ. 1
6.7 μ l	H ₂ O	3 PCR2.1 + BEV clone #3	Univ. 1
20.0		4 "	Univ. 1

Program #67 (9600 PE machine)

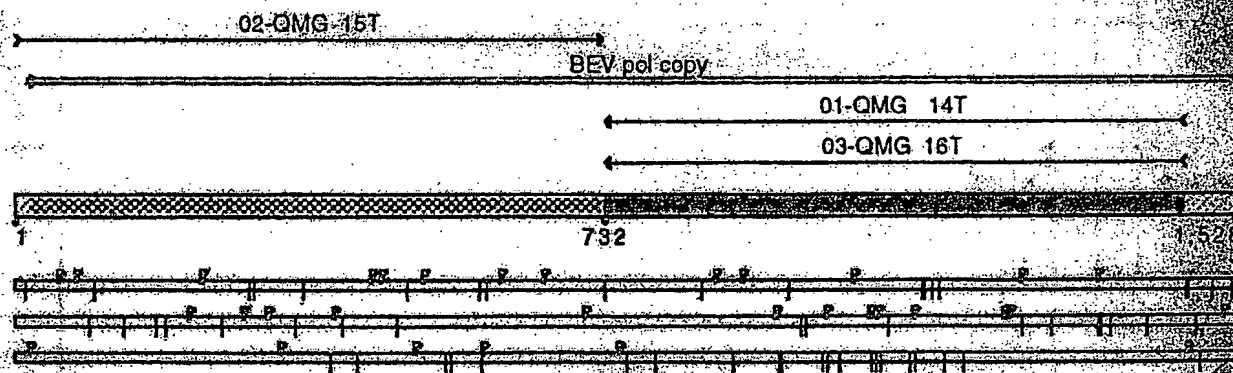
Cleaned up using 2 μ l 8M Na acetate, pH 5.2 + 50 μ l 100% ethanol
spin 14K/4°C for 20', 70% wash, dry pellet.

	Rxn
OMG 14	1
15	2
16	3
17	4

Results: sequences downloaded to Sequencer, aligned
with original BEV polymerase sequence, contig formed

Contig [0006]

Sequencher™ "PCR2.1 + BEV analysis"



OMG 15T did not align into contig; analysed individually
PCR2.1 + BEV clone #1 mutation in sequence within
primer BEV-1 (AA instead of AG)
PCR2.1 + BEV clone #3 mutation in sequence within primer
(AA instead of AAA)
PRIMER BEV-15 CGGCAGATCTAA CAA TGGCAGGAC AATA TCG AGT A

Continued on

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Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Cavanagh

M. Bernard 12/2/98

V. Hardy 12/2/98

M. Cavanagh

Signed

Dated

Signed

Dated

11/2/98

Set up sequencing reactions: USING BIG DYE TERMINATOR
MAX

PCR2-HBEV polymerase, clones #1, #3, #4

new primer designed to check for ambiguities from
sequencing of results obtained with univ. F+R primers
(p. 34)new primer is BEV-5, conc: 15 μ g MW 7079.6

5' TTC TTG TGG AGG ACA GCC GGT TC 3'

$$\frac{15 \mu\text{g}/210 \mu\text{L}}{7079.6} \times 10^6 = 102 \mu\text{M}$$

diluted to 10 μ M to set up
sequencing, stocks stored
@ -20°C.

DNA	4.0 μ l (mini prep Qigen spin column)	
Big Dye	8.0	see p. 10 for gel photo
10 μ M primer	0.3	of DNA extract with BM/Bg11
H ₂ O	7.7	
	20.0	

SEQ. RXN		label	
1	PCR2-HBEV #1	OMG-23	Program 67 (9600 PE machine)
2	" "	#3	" 24
3	" "	#4	" 25

11/12/98

Cleaning up seq. rxns:

20 μ l seq. rxn + 2 μ l 3M Na acetate + 52 μ l 95-100% ethanol
 ↓
 -20°C | ~20'
 ↓
 14K | 4°C | 20' remove SN
 ↓
 2 x 70% ethanol washes
 ↓
 pellets dried

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Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. Graham

Dated 11/2/98

Signed V. Hardy 11/2/98

Signed M. Graham 11/2/98

Dated

Signed

Dated

Signed

Dated

Sequencing cont. pEGFP+BEV clone #1

Date

11/2/98

Continued From

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Aim: to sequence pEGFP+BEV polymerase clone #1 from pool 3 (p. 33) using oligos designed for pEGFP-N1 vector

location of primers:

PRIMERS (resuspended in MQ. H₂O):

SV40r. seq

132 µg/210 µl mm = 102 µM

MW 6188

5' TTA TGT TTC AGG TTC AGG GGC 3'

GFP r. seq

132 µg/200 µl mm = 101 µM

MW 6095

5' CTG AAC TTG TGG CCG TTT AC 3'

CMV. seq

132 µg/210 µl mm = 101 µM

MW 6230

5' CGG TGG GAG GTC TAT ATA AG 3'

SET UP SEQUENCING REACTIONS: All primers diluted to 10 µM

DNA 4.0 µl (Qiagen miniprep DNA) see p. 33

IQMPRIMERX 0.3

BIG DYE 8.0

H₂O 7.7

20.0

Reactions run with others on p. 35
clean up as on p. 35

label SEQ. RXN

OMG 26 4 pEGFP+BEV #1

OMG 27 5

OMG 28 6

PRIMER

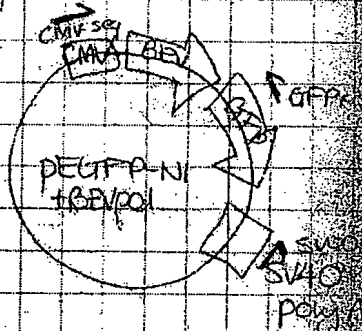
SV40

GFP

CMV

→ only 10 µl as tube gets
when setting up reactions

sequencing results analysed → confirms mutation in clone



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Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Beknard

V. Hardy

M. Guahan

M.B.
Signed

11/2/98
Dated

V. Hardy
Signed

11/2/98
Dated

17/2/98

Cloning to be done soon:

construct

primers

template

PCR BEV 2

BEV-1 + BEV-3

pK277 42

PCR BEV 3

BEV-4 + BEV-3

"

PCR Bam-GFP Bgl II

GFP-Bam + Bgl-GFP

PEGFP-NIMCS

PEGFP-NIMCS

pCMV.cass

N/A

PEGFP-NIMCS

Pin A1/NotI

Blunt end fill

religate.

Primers resuspended in MQ-H₂O

GFP Bam

17 μ g

MW 7893.2

STOCK CONC.

210 μ l103 μ M

Bgl-GFP

17 μ g

MW 7983.2

215 μ l100 μ M

BEV-3

108 μ g

MW 8928.8

120 μ l100 μ M

BEV-4

105 μ g

MW 8600.6

125 μ l98 μ M

PCR not set up, as decided to set up sequencing of PCR2.1+BEV #4 (universal forward/reverse primers) as results of sequencing analysed today for clones #1 + 3. See p. 38 for details.

46

32

Conducting Experiment:

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Morley

M. Greenham

Bernard 17/2/98

V. Morley 17/2/98

M. Greenham 17/2/98

Dated

Signed

Dated

Dated

SEQUENCING : PCR 2.1 + BEV clone #4

Date
17/2/98

Continued From

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Aim: to sequence clone #4 as results of sequencing with BEV-S primer have confirmed mutation in clones #1 + #3 (see p. 39)

Method

BIG DYE	8.0 μ l	
DNA	4.0	Miniprep, Qiagen spec
10 μ M primer	0.3	
H ₂ O	6.7	
	<u>20.0</u>	program 67
		cleaned up as on p. 35

Reaction	label	primer
1	DMG29	universal forward
2	DMG30	universal reverse

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Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Cuthbert

M. Bernard
Signed

17/2/98
Dated

V. Hardy
Signed

17/2/98
Dated

SEQUENCING RESULTS WITH BEV-5 PRIMER

Page

39

Date

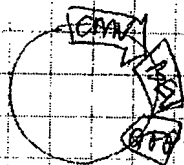
17/2 98

Continued From

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Aim: to analyse results from sequencing of PCR2.1 + BEV clones #1, 3 and 4 with BEV-5 primer.



Primer designed approx 100bp within BEV polymerase (reverse direction)

BEV pol Translated Sequence
Monday, 8 December 1997 3:13 PM

Page 1

Sequence Range: 1 to 1371 BgII

BEV-5: CGG CAG ATC TAA CAA TGG CA

3' → 5' ATGACGAT CGACGATG GCGAGGCA AGATGCGAG ATGACGATG ATGACGATG
3' → 5' ATGACGAT CGACGATG GCGAGGCA AGATGCGAG ATGACGATG ATGACGATG
OlyGluHis, OlyGluHis, OlyGluHis, OlyGluHis, OlyGluHis, OlyGluHis, OlyGluHis, OlyGluHis

End of primer BEV-1

5' CCACCCAGAC TAAGCTAGAA CCGAGGTTT TCTGAGGCT CTTCGCGGT GTTAAAGT
CUTGCTGCTG ATTCGATCTT GCGGCGAAA AGAAGCTGCA GAGGCGCCA CAATTGCGT
ProHisGlnHis LysLeuGlu ProSerVal PhePheAspVal PheProGly VallyGlu

F. BEV-5 PRIMER

5' CCGCTGCTG CCGAGGCA GACAGAGGC TTGAGACAA CTTCGAGAA GCGCTTTT
ProHisValLeu HisLeuGlu AspLysArg, LeuGluHisHis PheLeuGlu AlaLeuPhe

Sequences checked → go ahead with cloning using #4 17/2/98 this book.

Results: comparison to reverse complement sequence (starting PCR2.1 + BEV #1 at end of BEV primer in reverse direction)

expected: 5' ~~CGG CAG ATC TAA CAA TGG CA~~

actual: ✓✓✓ (X) ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

A mutation

confirms result from p.34

sequence is AA instead of ACA within primer region of BEV-1

PCR2.1 + BEV #3 (compared to above expected sequence)

actual: ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ (X) ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

mutation

confirms result from p.34

sequence is AA instead of AAA within primer region of BEV-1 (sequence highlighted above)

PCR2.1 + BEV #4 is in opposite orientation? oops looking at universal primer

actual: ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

analysis: ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓ ✓✓✓

no mutations with clone #4 ∴ check universal

and forward & reverse sequences (set up on p.38) PRO P.40

Bernard

J. Hardy

M. Graham

17/2 98

17/2 98

17/2 98

Dated

Signed

Dated

Signed

Dated

NEW CLONING: PEGFP + BEV (clone #4 of PCR2-HBEV)

Date

18/2/98

Continued From

Page #:

Book #:

Aim: - to use PCR2-1 + BEV clone #4 to clone into pEGFP-N1
 - results from p.39 suggest clone #4 doesn't have any mutations in the BEV-1 primer region whereas clones #1 and #3 have mutations

Method:

insert

PCR2-1 + BEV #4 (13.1.98) Qiagen mini DNA	15.0 μ l
BM 10x buffer M	8.0
BM Bgl II	1.0
" Bam HI	1.0
H ₂ O	28.0
	50.0

vector

PEGFP-N1 / Bgl II / Bam HI

DNA remaining from 2.2.98 and also SAP treated
 DNA from 26.1.98

inc 2 hr @ 37°C

precipitation of insert DNA

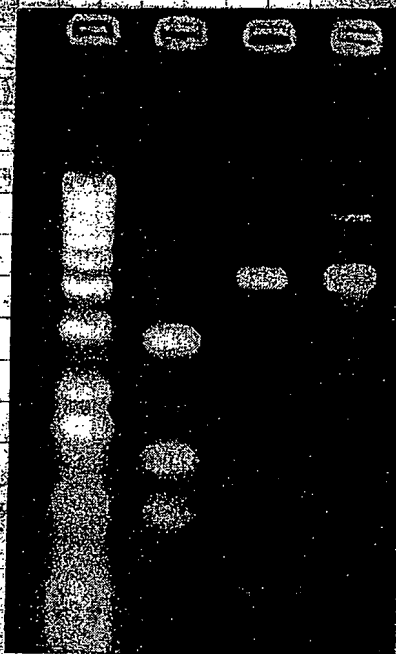
5 μ l 3M Na acetate + 12.5 μ l 100% ethanol + 5 μ l digest
 spin 20' 14K @ 4°C
 70% ethanol wash & resuspended in 20 μ l H₂O
 aliquot run on gel with vectors

1% TAE agarose gel

- 1 5 μ l 1kb ladder
- 2 2 μ l PCR2-1 + BEV #4 Bgl II / Bam HI
- 3 PEGFP Bgl II / Bam HI (2.2.98) 1 μ l
- 4 " " + SAP (26.1.98) 1 μ l
- 5 -

LIGATIONS

	-SAP	+SAP
PEGFP-N1 / Bgl II / Bam HI	2.5 μ l	5.0 μ l
insert	4.0	4.0
10x ligation buffer	1.0	1.0
ligase	1.0	1.0
H ₂ O	1.5	-
	10.0	11.1



Continued on

Page #:

Book #:

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

J. Hardy

M. Graham

MJB

19/2/98

J Hardy 19/2/98

M Graham

Signed

Dated

Signed

Dated

10/12/98

Aim: - to transform ligation from yesterday (p.40)
into DH5α

Method: -

- electocompetent DH5α, 50μl aliquots
electoporated into DH5α (2000V, 25μF, 2.5kV Biorad Gene Pulser)
- | | | | | |
|-----|---------------------|-----|-----|-----|
| (1) | P. EGFP + BEV - SAP | 3μl | 43 | kan |
| (2) | P. EGFP + BEV + SAP | 3μl | 43 | kan |
| (3) | pUC18 (50pg/μl) | 1μl | 4.6 | Amp |

Time constants.

Recovered in SOC, plated out onto kan or Amp plates

(1) 100μl + 200μl plated out, 250μl remaining

(2) 100 200 " " 550μl "

(3) 50μl " " 250μl "

inc. 37°C 1N → results: lots of colonies!
control ok.

11/2/98

COUNTERSELECTION FOR KAN^R COLONIES

colony #

1-24 = PEGFP + BEV + SAP

25-90 = PEGFP + BEV

colonies streaked onto both kan + amp plates, incubated
on bench (2 days)

20/2/98

RESULTS FROM COUNTERSELECTION

colony #

1-24

kan^c

Amp^R

36

"

"

38, 39

"

"

48

"

"

51

"

"

67

"

"

74

"

"

81

"

"

89, 90

"

"

all other colonies kan^R only

2-3ml LB + kan cultures

set up for minipreps (pools)

pool (colony #)

pool (colony #)

25-30

68-72

31-35

73-78 (-74)

37-42 (-38, 39)

79-84 (-81)

43-47

49-54 (-51)

55-60

61-66

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Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. C. Graham

Bernard 22/2/98

Dated

V. Hardy

Signed

22/2/98

Dated

M. C. Graham

Signed

25/2/98

Dated

6
POOLED MINIPREPS : PUTATIVE PEGFP + BEV CLONES

Date

23/2/98

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Diagen minis, spin columns
eluted in 100ul MQ H₂O
digested 12.0ul with BamHI / BglII
x10

DNA 12.0ul		
BM	10x Buffer	2.0
	BglII	0.5
	BamHI	0.5
	H ₂ O	5.0

80.0 → 8.0ul per tube

+ DNA

inc. 37°C / 15 hours

1. Agarose TAE

600ng
1kb

25-30

31-35

37-42

43-47

48-54

55-60

61-66



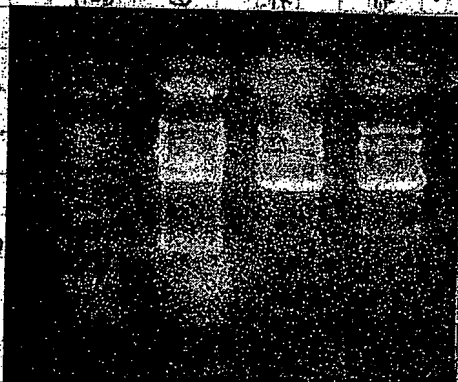
← 1.4kb BEV poly

600ng
1kb

24-29

33-38

50-54



1.0kb →

← 1.4kb

4 pools have a 1.4kb band (BamHI/BglII fragment)

25-30

31-35

61-66

79-84

} single

minis set up (2ul LB+kan 100mg/ml)

25, 61, 62, 63, 64, 65, 66, 79, 80, 82, 83, 84

inc. 37°C O/N

Continued on

Page #: 44

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Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Hardy

M. Gualto

M. Bernard 23/2/98

V. Hardy 23/2/98

M. Gualto

Signed

Dated

Signed

Dated

SEQUENCING RESULTS : PCR2.1 + BEV clone #4

Date

23/2/98

Continued From

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Results:

PCR2.1 + BEV #4 FORWARD PRIMER @MG29

- signal quite low, only some readable sequence
- BEV-1 primer sequence located and checked → OKVV
- sequencing artefacts particularly in GGT regions

PCR2.1 + BEV #4 REVERSE PRIMER @MG30

- signal a bit bigger, more readable sequence
- BEV-2 primer sequence located and checked → VV OK
- some of BEV polymerase sequence checked → VV OK
- sequencing artefacts → T region, GGT region, but peaks underneath are readable and obviously present.

Comments:

Cloning with PCR2.1 + BEV #4 should work!

Next: check single minis from pools, set up today.

Continued on

Page #:

Book #:

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

M. Cavanagh

B

23/2/98

Hardy

23/2/98

Cavanagh

23/2/98

Dated

Signed

Dated

Dated

MINIPREPS FROM POOLS : pEGFP + BEV

Date

24/2/98

Continued From

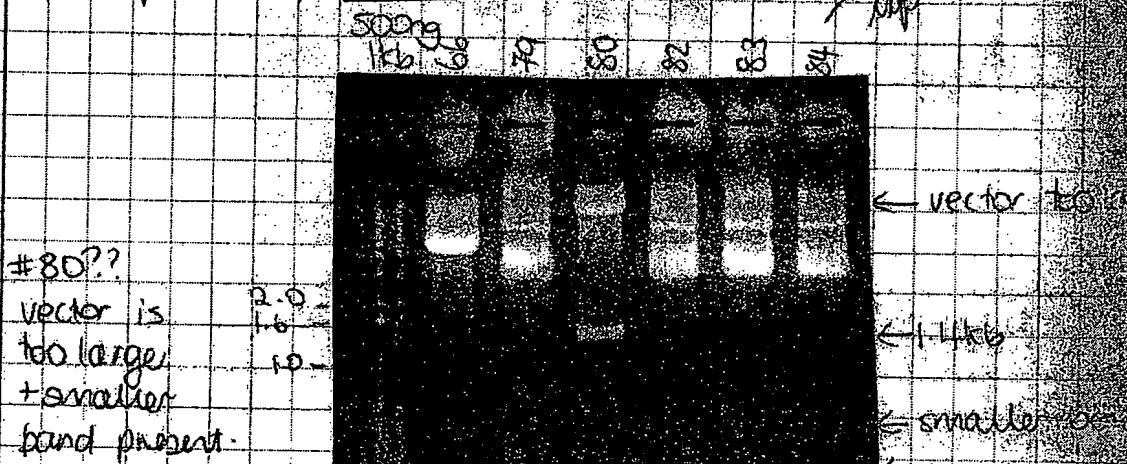
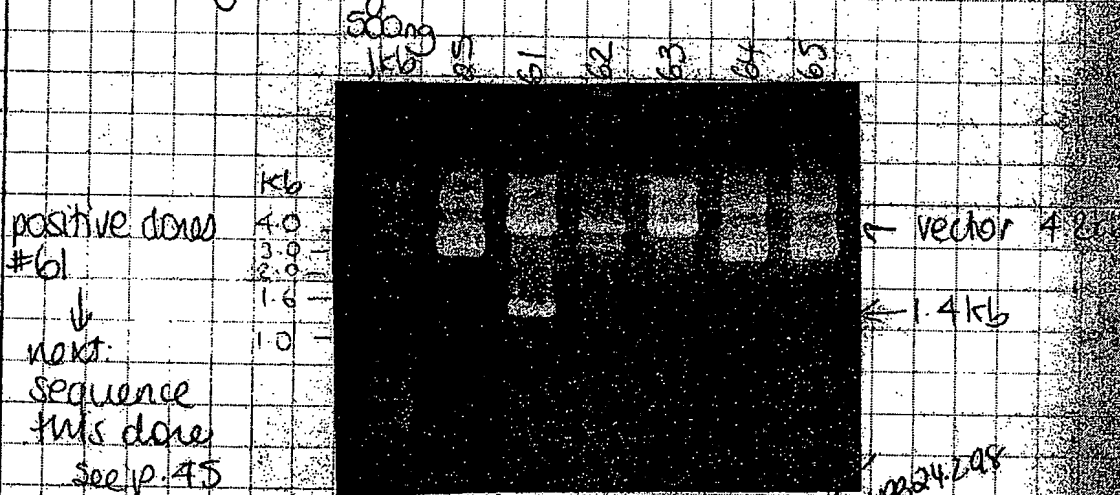
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Qiagen minis, 1.5ml culture
 eluted in 100ul MQ H₂O
 digested 5.0ul with BamHI + BglII to release 1.4kb
 BEV polymerase fragment.

DNA	5.0ul	x12	
BMI 10X (M)	2.0	24.0	
" BglII	0.5	6.0	
" BamHI	0.5	6.0	
H ₂ O	12.0	144.0	
	20.0	180.0	→ 5ul x12 + 5ul DNA inc. 37°C/1.5hrs

1/ TAE agarose gel



Continued on

Page #

Book #

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

V. Maray

M. Guadalupe

M. Bernard

24/2/98

V. Maray

24/2/98

M. Guadalupe

Signed

Dated

Signed

Dated

SEQUENCING : PEGFP + BEV clone #61

Date

2/12/98

Continued From

Page #: 44

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Aim: to sequence clone #61 with primers designed for pEGFP vector

Method: used Big Dye Terminator mix

primers: as on p. 36 10 μ M aliquots
 SV40 r-seq \rightarrow GFP sequence / SV40 junction
 GFP r-seq \rightarrow look for GFP + BEV pol. (3' end)
 CMV-seq \rightarrow look for BEV polymerase (5' end)

mini prep DNA (p. 44) lane 3 of top gel, clone #61

		1	2	3
		GFP	CMV	SV40
BIG DYE	8.0 μ l	✓	✓	✓
DNA	4.0	✓	✓	✓
10 μ M primer	0.3	✓	✓	✓
H ₂ O	7.7	✓	✓	✓
	20.0			

Method 67 (9600 PE)

Clean up

0.1 vol Na acetate, 2.5 vol 95% ethanol, 20°C / 1 hour
 14K 125' @ 4°C
 2 x 70% ethanol washes
 pellets dried

QMG+	31	PEGFP + BEV clone #61	GFP r-seq
"	32	"	CMV-seq
"	33	"	SV40 r-seq

Continued on

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Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

Bernard 2/12/98

Dated

V. Hardy

Hardy 2/12/98

Signed

Dated

U. Cressen

Cressen 3/3/98

Signed

Dated

27/2/98

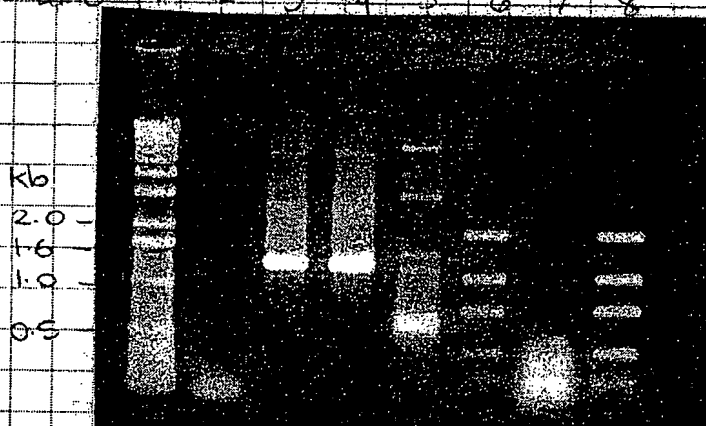
PCR reactions from p. 46
1% TAE gels

lane

- 1 1 µg 1 kb ladder (Gibco)
- 2 10 µl reaction 2 = PWV WORK!
- 3 10 µl " 3
- 4 " 4
- 5 " 5
- 6 1 µl DNA mass ladder (Gibco)
- 7 10 µl PCR rxn 4 PWV (20.2.98)
- 8 2 µl DNA mass ladder

80V

lane 1 2 3 4 5 6 7 8



mass ladder

1 µl 2 µl

ng DNA ng DNA

50 100

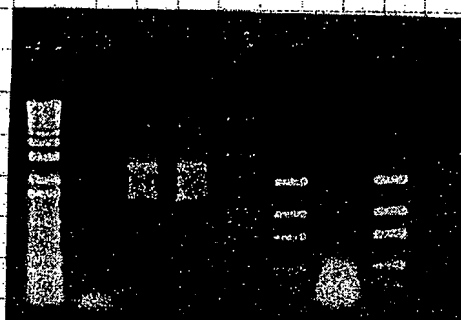
30 60

20 40

10 20

5 10

>100 ng of each PCR product obtained (in 100 µl aliquots)



bands from lanes
3, 4 & 5 cut out
for gel purification

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

V. Hardy

C. Graham

Bernard 27/2/98

V. Hardy 27/2/98

27/2/98

3/3/98

Signed

Dated

Signed

Dated

Signed

Dated

Gel purification of PCR products:

Date

2/3/98

Continued From

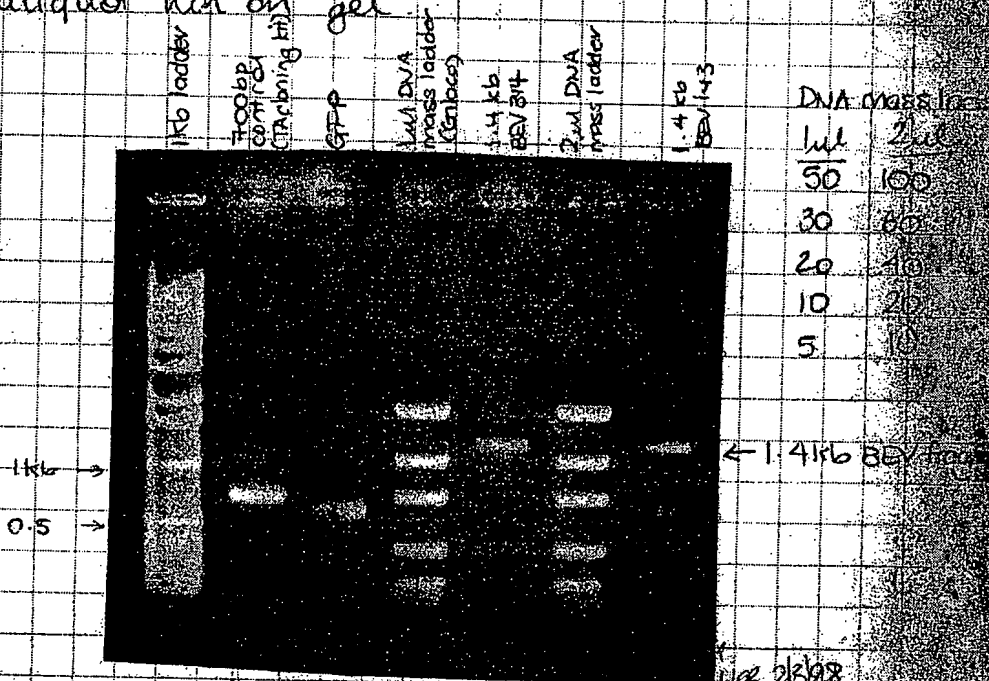
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Gel purification using Qiagen spin columns:

700bp control band (TA cloning kit) $1.39g - 1.02g = 0.37g$ ~700bp GFP band (Bam-GFP+GFP-Egl product) $1.14g - 1.03g = 0.11g$ 1.4kb BEV band (BEV3+4 product) $1.12g - 1.025g = 0.095g$ 1.4kb BEV band (BEV1+3 product) $1.12g - 1.026g = 0.094g$ 3x buffer QCR, all eluted in 30ul H₂O, speedvac 15 min

1ul aliquot run on gel

SET UP LIGATIONS INTO PCR2.1 (TA CLONING KIT-INVITROGEN)
approx. conc. from gel:

- ① GFP 5-10ng/ul
- ② BEV3+4 5-10ng/ul
- ③ BEV1+3 5-10ng/ul
- ④ 700bp control 20ng/ul

PCR2.1	2.0ul	control	2.0ul
10x buffer	1.0		1.0
insert	3.0		2.0
ligase	1.0		1.0
H ₂ O	3.0		4.0
	10.0		10.0

Inc 14°C o/n

Continued on

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Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

P. Campbell

M. C. C. C.

Signed

Dated

Signed

Dated

2/3/98

02/03/98

Transformations : pOR2.1 ligations
(GFP, BEV143, BEV344
TA cloning kit control)

Date

3/3/98

Continued From

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Aim: to use kit cells (TOP10E)
heat shock protocol

Method: as per TA cloning kit instructions.

3ul of each ligation

plated 100ul ~200ul onto LB+Amp100
(actually <200ul)

inc. 37°C O/N

3/3/98

Results:

# colonies		blue	white	
700bp control		89	2	→ low. no. of white colonies
GFP	100ul	80	>100	→ 39 colonies picked 12 screened
	~200ul	>100	>100	
BEV143	100ul	>100	>100	→ 42 colonies picked #1-42
	200ul	>100	>100	
BEV344	100ul	>100 (some light blue)	~30	→ 42 colonies picked #43-84
	200ul	>100	>30	

Next: PCR screening of white colonies.

Continued on

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Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

Bernard 4/3/98

Dated

Campbell

Signed

9/10/98

Dated

M. Graham

Signed

4/3/98

Dated

Date

A/3/98

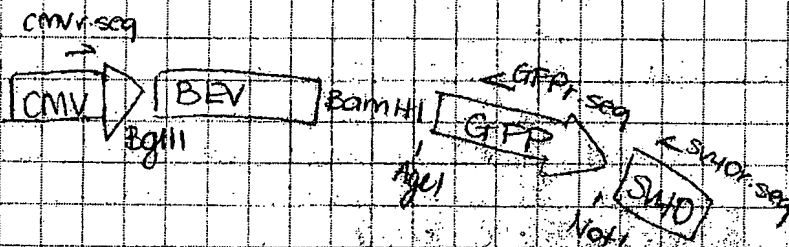
Continued From

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SEQUENCING RESULTS: PEGFP + BEV clone #61
(~~EGFP-BEV-1~~) PEGFP-BEV-1

sequencing results for: DMG 31 GFP_r seq
DMG 32 CMV seq
DMG 33 SV40r seq



downloaded seq from hot shots to sequencer
looked for BamHI or BglII sites or NotI site, depending
on primer.
(no printouts from AGRF)

GFP_r seq result:

vector sequence - MCS of PEGFP - BEV-2 primer - BEV
polymerase sequence
sequence edited and checked → ok ✓

CMV seq result:

vector sequence - BEV-1 primer - BEV polymerase
sequence edited and checked → ok ✓

SV40r seq result:

located NotI site, have not checked 3' end of GFP seq

Next:

prepare more DNA of clone #61 for transfections
glycerol stock prepared on 6/3

Continued on

Page #:

Book #:

Name of Person Conducting Experiment

M. Bernard

First Witness of Experiment

P. Campbell

Second Witness of Experiment

M. Graham

M. Bernard

Signed

A/3/98

Dated

Campbell

Signed

A/3/98

Dated

M. Graham

Signed

PCR screening PCR2.1 + GFP

BEV143

BEV344

Date

5/3/98

Continued From

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Book # 32

Aim: to screen 12 white colonies of each of above transformations

Method:

PCR master mix

		x 39	
10 μ M Forward	0.2 ml	7.8 ml	
10 μ M Reverse	0.2	7.8	
10x PCR buffer 1Mg	2.0	78.0	(83005520-22)
Taq (31 Jan 99)	0.2	7.8	
50 mM dNTP's (Invitrogen)	0.1	3.9	
H ₂ O	17.3	674.7	
		780	→ 20 μ l per tube

colonies picked (yellow tip) → streaked onto fresh Amp plate
→ tip touched into PCR mix

Program #26 (9600 PE machine)

94°C 1:30
94°C 0:30
55°C 0:45 } x35
72°C 1:00
72°C 5:00
15°C ∞

PCR reactions

1-13 20 μ l plate GFP colonies #1-12 #13 = blue
14-26 " BEV143 colonies #14-25 #26 = blue
27-39 " BEV344 colonies #27-38 #39 = blue

15 μ l of each PCR reaction run on 1% TAE gel
(except #39 - not enough wells)

Continued on

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Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

Bernard 5/3/98

Dated


Signed

95/03/98

Dated


Signed

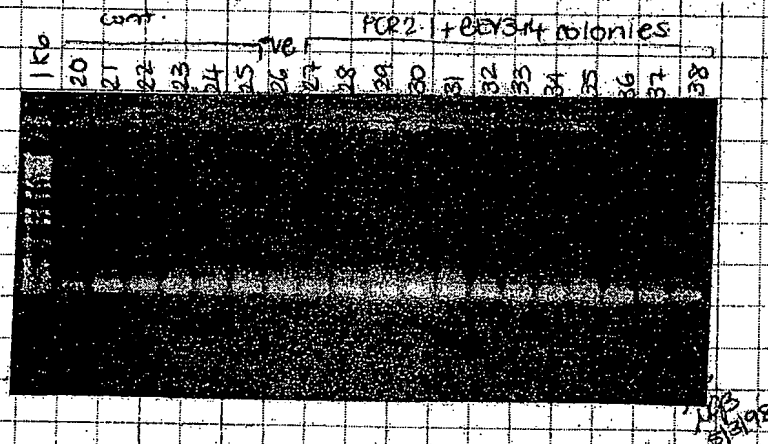
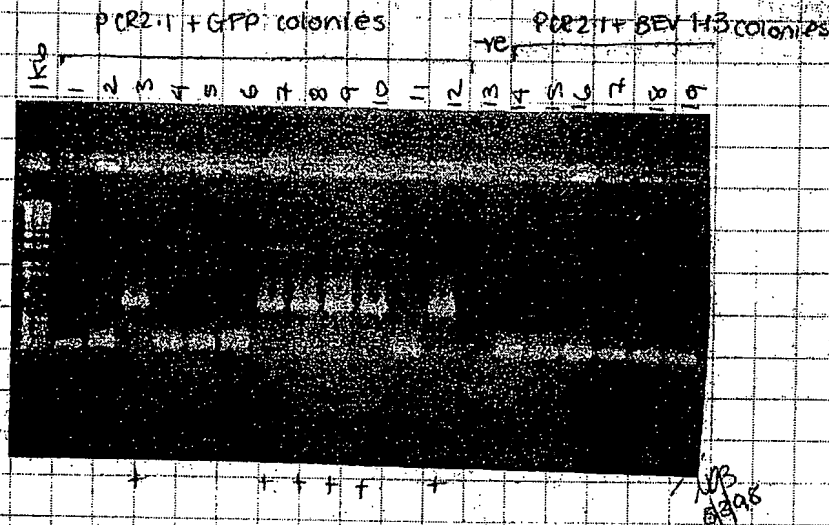
5/3/98

Dated

5/3/98

PCR screening results : GFP
BEV 1+3
BEV 3+4

1% TAE agarose gels



Comments:

6/12 positive for PCR2.1 + GFP → set up minis

0/12 " " PCR2.1 + BEV 1+3 → set up pooled minis

0/12 " " PCR2.1 + BEV 3+4 → set up pooled minis

LB + Amp cultures inc. O/N @ 37°C

Minis set up of 3, 7, 8, 9, 10, 12 (GFP)

Pooled minis 7 x 6 colonies of BEV 1+3, BEV 3+4 = 42 colonies streaked onto numbered plate

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Berrand

D. Campbell

M. Cribban

M. Berrand 5/3/98

Signed

Dated

Signed

05/03/98

Dated

24

MINIPREPS : SINGLES OF PCR2.1 + GFP

POOLS OF PCR2.1 + BEV143

" " " 18EV344

Date

6/3/98

Continued From

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Aim: to prepare mini-DNA for digests of above.

Method: Qiagen spin columns

1.5ml culture

eluted in 100ul H₂O

set up digests

POOLS 14

x14

(7 pools PCR2.1 + BEV143

7 pools PCR2.1 + BEV344)

see below for colony #

DNA 12.0ul

10x buffer 2.0 28.0

BM ECOR1 1.0 14.0

H₂O 5.0 70.0

20.0 112.0

larger aliquot of ECOR1 used

exp. 31 Mar 98

pools identity

+ BEV143

BEV344

① 1-6 43-48

② 7-12 49-54

③ 13-18 55-60

④ 19-24 61-66

⑤ 25-30 67-72

⑥ 31-36 73-78

⑦ 37-42 79-84

GFP x6

x7

DNA 5.0ul

10x buffer 2.0 14.0

ECOR1 1.0 7.0

H₂O 12.0 84.0

20.0 105.0

→ 12.0ul per tube instead of 15.0ul
clones # 3, 7, 8, 9, 10, 12.

1.5 hours @ 37°C

15ul of digest loaded onto 1% TAE agarose gels.

PCR2.1 + GFP ~~clones~~ ^{minis} digested with ECOR1 (first lane undigested DNA, second lane ECOR1 digest)

1x6 #3 #7 #8 #9 #10 #12

← insert
b/w 500-1000bp

correct sized inserts with all minis

next: sequence → see p. 60

PTO for pooled minis / digests

Continued on

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Book #: 32

Bernard

P. Campbell

M. Graham

Bernard

6/3/98

P. Campbell

06/03/98

M. Graham

6/3/98

Dated

Dated

Dated

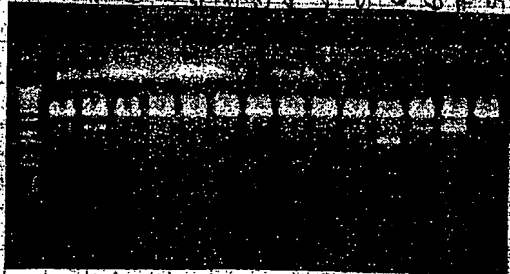
Dated

6/3/98

MINIPREP DIGESTS CONT: PCR2.1 + BEV POOLS

ECORI digests of PCR2.1 + BEV pools
 1% TAE agarose gel
 90V/35', stained in EtBr.

PCR2.1 + BEV143					PCR2.1 + BEV314				
1-0	2-1	3-18	4-24	5-30	6-0	7-60	8-66	9-72	10-78



← 2.0kb insert
 ← 1.4kb insert

PCR2.1 + BEV143 → only one pool (7-12) is showing insert band, but it is too large

PCR2.1 + BEV314 → pool 61-66 has an insert band ~1.4kb (could be larger?)

NEXT: set up single min. cultures from pool 61-66
 screen more colonies from PCR2.1 + BEV143

Name of Person Conducting Experiment

M. Bernard

First Witness of Experiment

P. Campbell

Second Witness of Experiment

M. G. Gable

mpb
 Signed

6/3/98
 Dated

phw 06/03/98
 Signed

Dated

MIDIPREPS: - PEGFP + BEV clone#61

- ~~PCR BEV 1~~ in patent P EGFP BEV.1 in patent

- PEGFP - NI MCS

Date

9/3/98

Continued From

Page #:

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Aim: to prepare larger amounts of DNA for mammalian cell transfections.

Method:

starter cultures: incubated 37°C ~ 6 hours

PCR BEV.1 (clone#61)

- culture from glycerol stock still in fridge (6/3)

4ml LB+kan50 + 0.5ml culture from fridge

PEGFP-NI-MCS

- glycerol stock (250µl) + 2ml LB+kan50

25ml cultures: used starter culture to inoculate following:

2x25ml (set up 75ml LB+kan) clone#61

1x25ml (set up 50ml LB+kan) PEGFP-NI-MCS

37°C O/N

10/3/98

Qiagen midi prep kit

PEGFP + BEV clone#61 (= PCR BEV.1)

→ 2x30ml

} cells harvested in oaridge tubes 8K/5' @ 4°C

PEGFP-NI-MCS

→ 2x25ml

↓
kit protocol followed

↓
DNA ppt'd in corex tubes (2 tubes per DNA)
10K/30' @ 4°C

↓
70% wash ethanol (2ml/tube)

↓
pellet air dried 20'

↓
resuspended in 100µl MQ H₂O
stored @ 4°C O/N

Continued on

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Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

Bernard

10/3/98

Campbell

10/03/98

Graham

10/3/98

Dated

Signed

Dated

Dated

9/3/98

PCR2.1+BEV143 → screening of more colonies from

" BEV344 → minis #61-66 = PCR BEV.3

Aim: to set up miniprep cultures of above

Method:

minis 61-66 = PCR2.1+BEV344 singles
LB+kan 1-2ml cultures

pools 1-6 = PCR2.1+BEV143 white colonies

7-12 "

13-18 "

19-24 "

white & blue colonies

LB+kan 1-2ml cultures

37°C O/N

10/3/98

Qiagen minispin columns

1.5mls culture

eluted in 100ul MQ H₂OMiniprep digests

singles (61-66)

NB. #62 didn't grow

pools (x4)

DNA	5.0ul	x7
BM 10x buffer	2.0	14.0
BM EcoRI (10ul)	1.0	7.0
H ₂ O	12.0	84.0
	20.0	105.0

larger aliquot

7x15ul

DNA	12.0ul	x4
BM 10x buffer	2.0	8.0
BM EcoRI (10ul)	1.0	4.0
H ₂ O	8.0	60.0
	20.0	72.0

inc. 37°C 12 hrs.

15ul loaded onto 1% TAE agarose gel

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

P. Campbell

M. Graham

M. Bernard

10/3/98

Campbell

10/03/98

Signed

Dated

Signed

Dated

Digest results: ① PCR2.1 + BEV143 pools (= PCR.BEV.2
translatable BEV construct)
② PCR2.1 + BEV344 clones #61-66 (= PCR.BEV.3 non-translatable BEV construct)

Date

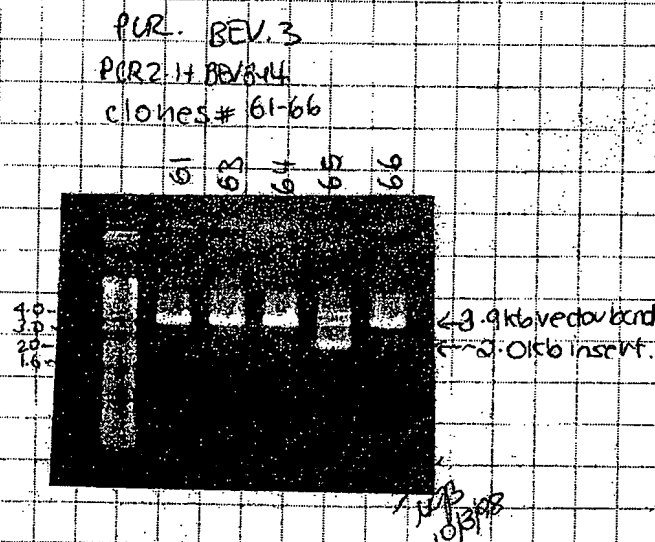
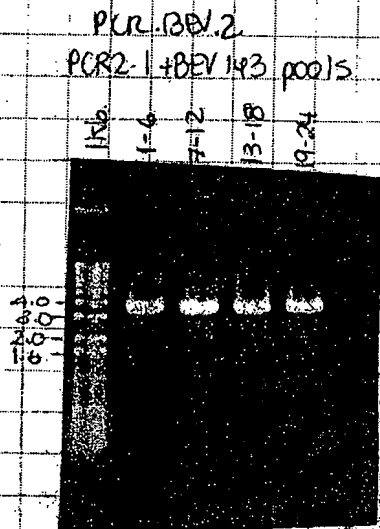
10/3/98

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nb. names of final constructs in brackets i.e. when in expression vector pEGFP-N1-MCS



Comments:

PCR2.1 + BEV143 pools : - no insert bands in this batch of 24 colonies

PCR2.1 + BEV344 clones 61-66: - insert band only with clone #65 but it is too large. Size looks greater than 2kb - a bit strange as band on previous gel (pool 143) looks < 2.0kb
- digests look a bit strange - could be due to old enzyme (exp. 31 March 98)

Next: - set up ligations again
- firstly clean up PCR products (use gel purifn columns)
- maybe repeat digests with a new ECOR1 stock?

Continued on

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Book #:

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

Bernard

10/3/98

Dated

Signed

10/3/98

Dated

10/3/98

Dated

11/3/98

MIDIPREP DIGESTS: ~~PER BEV1~~ and ~~PEGFP-N1-MCS~~
PEGFP BEV1

Aim: - to check midiprep DNA by digesting with BamHI
and/or BglII
- to determine concentration

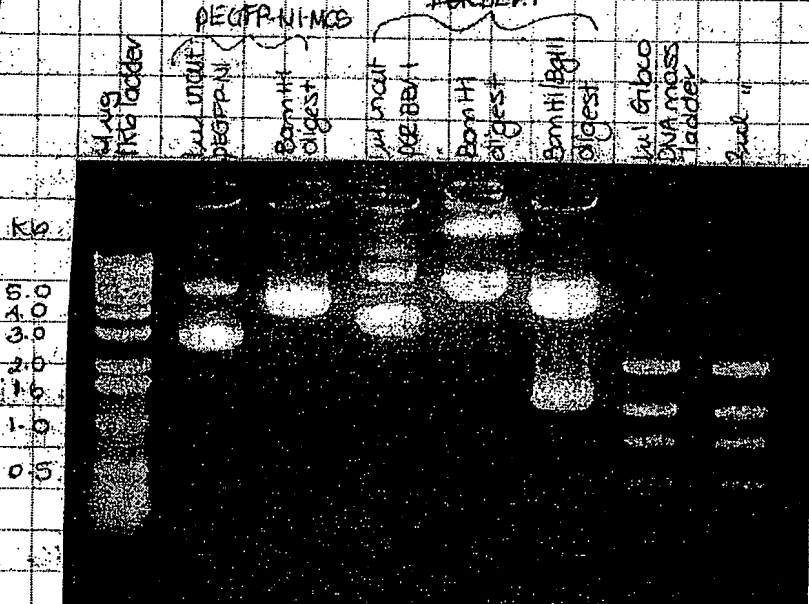
Method:

DNA from 4°C, 2x100µl aliquots combined & tubes
rinsed with an extra 100µl MQ-H₂O (final volume
~300µl)

Digests:

DNA	2.0µl	2.0µl	2.0µl	
10x buffer/M	2.0	2.0	2.0	
BamHI	1.0	1.0	1.0	100-37°C
BglII	-	-	1.0	2 hours
H ₂ O	15.0	15.0	14.0	
	20.0	20.0	20.0	

20µl digest run on 1% TAE gel along with 1.0µl
undigested DNA.



PEGFP-N1-MCS = 4.2Kb shown by BamHI digest

PEGFP BEV1

~~PER BEV1~~ = 9.6kb shown by BamHI digest

BEV1 insert = 1.4Kb shown by BamHI/BglII digest

concentration of both midipreps >100ng/µl

M. Bernard

P. Campbell

M. C. Graham

M. Bernard

P. Campbell

24/3/98

MIDIPREP CONCENTRATIONS

Date

11/3/98

Continued From

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spectrophotometry results:

1:60 dilution in $\text{Na}_2\text{H}_2\text{O}$.

11/3/98

1:60 diln
ng/ μl

NAME	A320	A280	A260	280/260	260/280	PROTEIN	NUCLEIC ACID
------	------	------	------	---------	---------	---------	--------------

PEGFP-N1-MK8

0.0041	0.1990	0.3297	0.5985	1.6709	55.945	13.463
--------	--------	--------	--------	--------	--------	--------

0.0085	0.3015	0.5408	0.5505	1.8167	51.642	22.976
--------	--------	--------	--------	--------	--------	--------

PCR-BEV1

PEGFP-BEV1

PEGFP-N1-MK8 = 800 ng/ μl
= 0.8 $\mu\text{g}/\mu\text{l}$

PCR-BEV1 = 1376 ng/ μl PEGFP-BEV1 = 1.4 $\mu\text{g}/\mu\text{l}$

11/29/98

JAB
11/3/98

Mick has used the DNA to transfect cells today.

Continued on

Page #:

Book #:

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

11/3/98

11/3/98

Campbell

11/03/98

M. Graham

11/3/98

Dated

SEQUENCING: PCR2.1 + GFP clone #3

Date

11/3/98

Continued From

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(E PCR-Bgl-GFP-Bam)

Aim: to check above clone by sequencing with universal forward & reverse

Method: sequencing reactions set up on 10.3.98
DNA of clone #3, digest shown on p. 53
100ul miniprep DNA

DNA 4.0ul
Big Dye 8.0
Lum primer 3.0
H₂O 5.0
20.0

Program 67 (9600 PE machine)

↓
Clean up: as per p. 45
only 1x 70% ethanol wash

QMG 36 F
QMG 37 R

Continued on

Page #:

Book #:

Name of Person Conducting Experiment

M. Bernard

First Witness of Experiment

P. Campbell

Second Witness of Experiment

M. Givhan

M. Bernard

Signed

11/3/98

Dated

P. Campbell

Signed

11/03/98

Dated

M. Givhan

Repeat of ligations: PCR2.1 + BEV143 (PCR.BEV.2)
 PCR2.1 + BEV3+4 (PCR.BEV.3)

Date

12/3/98

Continued From

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Aim: - to set up new ligations using cleaned up PCR product

- use PCR products → 80ul reactions originally set up on p.46 & only 10ul run on gel, which was gel purified for first ligation (see p.48)
- should be ~40ul remaining stored @ 4°C.

Rxn 3 BEV143
 4 BEV3+4

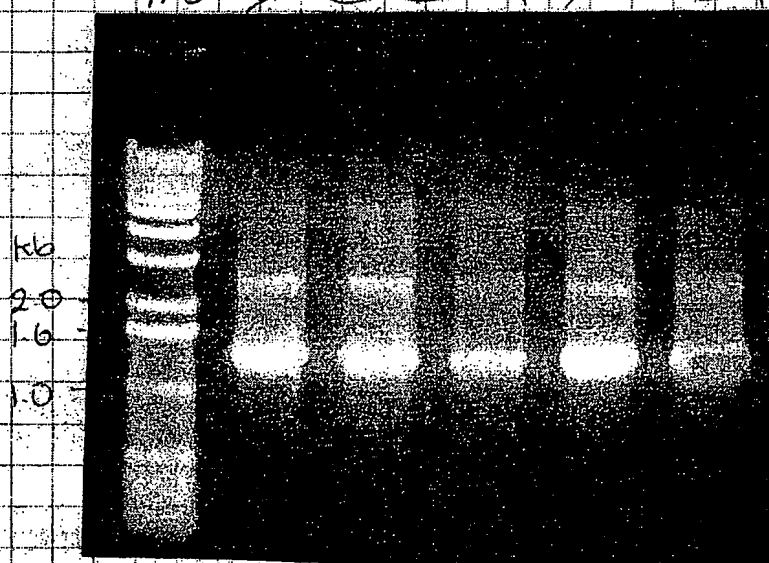
- could be a problem if not gel purified as there is multiple bands (top gel photo on p.49) lanes 3+4

Method:

Remainder of reactions 3+4 run on 1% TAE agarose gel

2ul PCR reaction (3)
 = BEV143

PCR reaction (4)
 = BEV3+4



← 1.4kb BEV PCR products bands out over incubated for gel purification

photo after excising bands.



← 1.4kb BEV products removed

Continued on

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Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

Bernard 12/3/98

Dated

Signed

12/03/98

Dated

12/3/98

Dated

12/3/98

LIGATIONS

PCR2.1 + BEV143 (1.4kb) = (PCR.BEV.2)

CONT.

PCR2.1 + BEV344 (1.4kb) = (PCR.BEV.3)

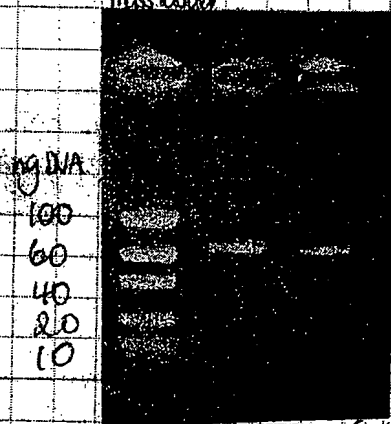
Gel purification of 1.4kb BEV PCR products:
using QIAquick columns

BEV143 = ③ wt. of bands ~ 0.3g x 2x buffer OG + 0.3ml isopropanol, eluted in 30ul

BEV344 = ④ wt. of bands ~ 0.25g x 3x buffer OG + 0.25ml isopropanol, eluted in 30ul

1ul aliquots of gel purified products run on

1% TBE gel
2ul DNA mass loader



Used Invitrogen TA cloning kit

PCR2.1 vector	2.0ul
10x buffer	1.5
Insert	7.5
H ₂ O	3.0
Ligase	1.0
	15.0

inc. 14°C O/N

13/3/98

Transformations into TOP10 cells from Invitrogen

5ul each ligation

① PCR2.1 + BEV143

② PCR2.1 + BEV344

Heat shock protocol, recovery in 250ul SOC (from Gibco)
Plated out 50-100ul onto LB+Amp+Xgal/100ul

Name of Person Conducting Experiment

M. Bernard

First Witness of Experiment

P. Campbell

Second Witness of Experiment

M. C. ...

M. Bernard

13/3/98

Campbell

13/03/98

...

Signed

Dated

Signed

Dated

PCR screening : transformations PCR2.1 + BEV.143 (=PCR BEV.2)

63

PCR2.1 + BEV3+4 (=PCR BEV.3)

Page

16/3/98

Continued From

Page #: 62

Book #: 32

Aim: to screen colonies for 1.4kb inserts.

PCR master mix for screening 20 white colonies of each tination:

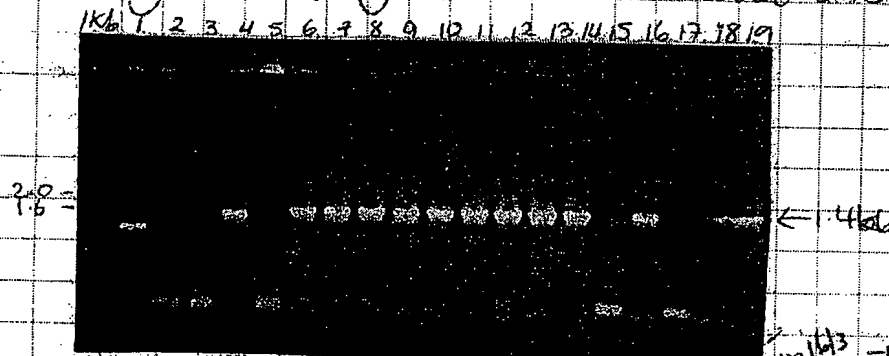
10x PCR+Mg buffer (tube 1)	2.0 μ l	116.0	x 58
25 μ M Tag	0.2	11.6	
16 μ M Forward primer	0.15	8.7	
16 μ M Reverse primer	0.15	8.7	
50 μ M dNTP's	0.10	5.8	
H ₂ O	17.40	1008.4	
	20.0	1160.0	20 μ l / tube

Green = BEV143 1-20 (incl. blue colony)
 Blue = BEV3+4 1-20 (incl. blue colony)

Program #26 9600 PE Plant lab

15 μ l of each reaction loaded on 1% TAE gel
 blue colony rxns (negative controls loaded onto another gel)

BEV143
 reactions
 1-19



positive colonies: (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+)

-ve controls
clone colonies

BEV3+4
 reactions
 1-19



positive colonies: (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+) (+)

app 143
163

Next: select colonies for setting up miniprep cultures
 O/N cultures set up (circled positive colonies above)

Continued on

Page #: 64

Book #: 32

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

B. Bernard

P. Campbell

M. Graham

MB

16/3/98

Campbell

16/3/98

16/3/98

Dated

Signed

Dated

Dated

MINIPREPS : PUTATIVE CLONES PCR2.1+BEV143 1.4kb (PCR)
 PCR2.1 + BEV3+4 1.4kb (PCR)

Date

17/3/98

Continued From

Page # 63

Book # 32

QIAGEN spin columns

1.5ml culture, eluted in 100ul H₂O

5ul DNA digested with EcoRI

DNA 5.0ul
 10X (H) buffer 2.0
 EcoRI 1.0
 H₂O 12.0
 20.0

clone #
 BEV143 4, 6, 7

BEV3+4 1, 5, 10

also included clone #65 (see p. 57)

repeated digest to check restriction

15ul digest run on 1% TAE gel

PCR2.1+BEV143

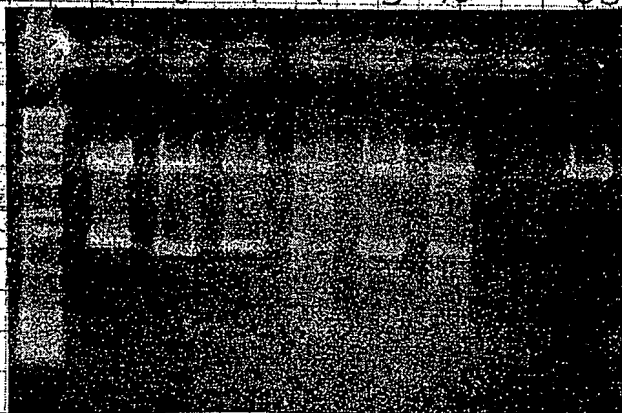
PCR2.1+BEV3+4

PCR2.1+BEV3+4

4 6 7

1 5 10

65



1.5
 2.0
 1.6

← 1.4kb insert

- all of the clones have a 1.4kb insert (except result on p. 57 with earlier digest of clone #65 is not)
- clone #6 of PCR2.1+BEV143 → insert looks slightly smaller than with clones #4 & #7.

Next: sequence clones with universal forward and reverse primers.

Continued on

Page # 67

Book # 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

P. Campbell

M. C. ...

M. Bernard

17/3/98

Campbell

17/03/98

M. C. ...

Aim: preparation of pCMV.eGFP

↓
pEGFP-N1-MCS NotI/PinAI deletion
fill in ends with Pfu polymerase

↓
religate

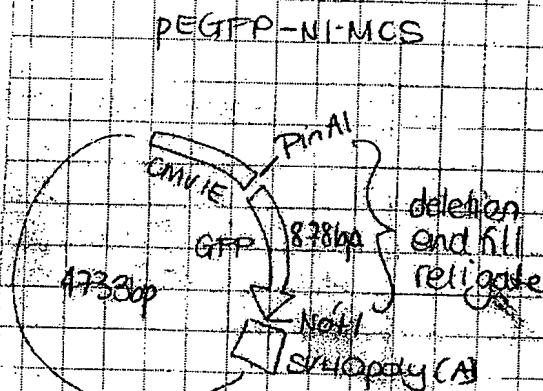
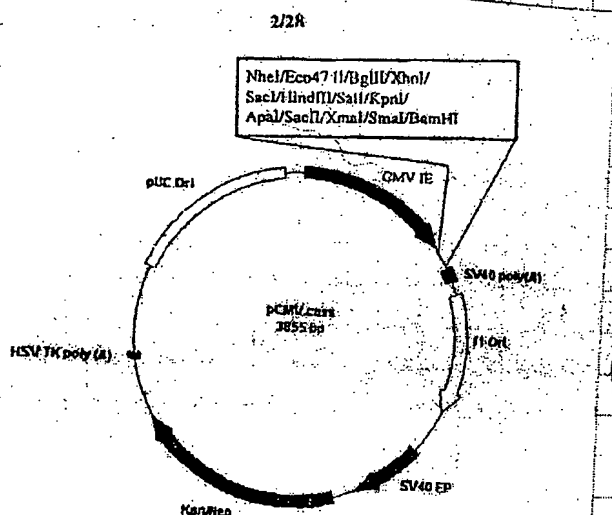


FIGURE 2

Method:

pEGFP-N1-MCS

DNA (mid) 15 µl inc. 37°C 2 hrs

NotI 1.5 µl

10x buffer H 5.0 µl

H₂O 28.5 µl

50.0 µl

Next digest: 14.5 µl DNA/NotI + 5 µl 10x buffer B, 1 µl cutal PinAI

+ 29 µl H₂O = 50 µl total

inc. 37°C 1.5 hrs

2 µl aliquot for gel

pot'd DNA 2.5 µl 100% ethanol 1/1000 Na.
acetate 1x70% wash
resuspended in 15 µl H₂O (0.5 µl for gel)

Continued on

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Book # 32

H. Bernard

P. Campbell

M. Cavanah

18/3/98

18/3/98

18/3/98

Dated

Stored

Dated

Dated

Date

18/3/98

Continued From

Page #: 65

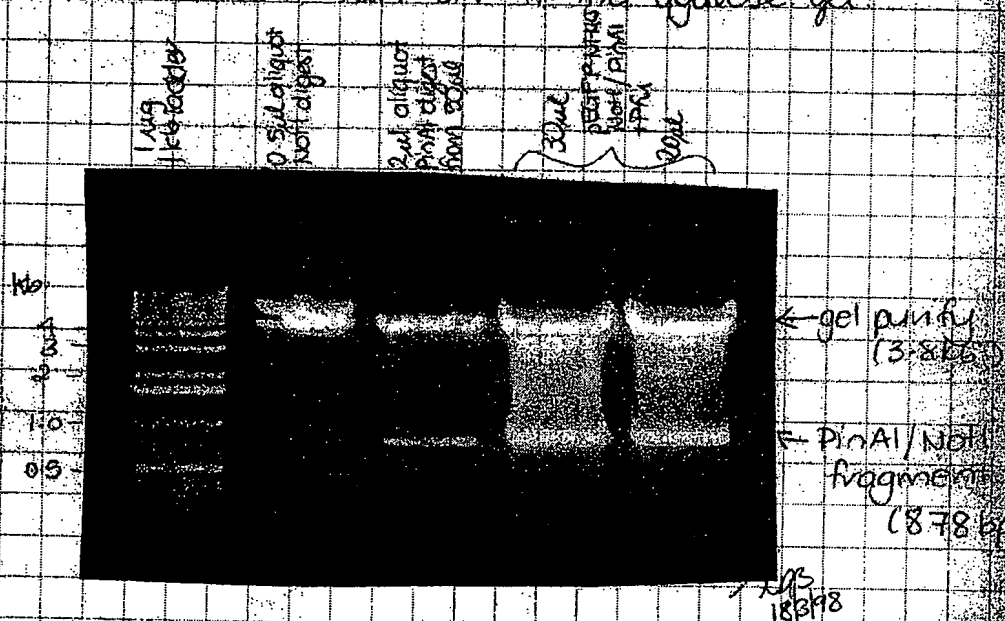
Book #: 32

Pfu treatment of pEGFP-N1-MCS /Not I + Pst AI

48 μ l digested DNA1 μ l 10MM dNTPs6 μ l 10X Pfu buffer (Stratagene)1 μ l Pfu polymerase " (donated Pfu pol)40 μ l H_2O 60 μ l total

72°C / 30 using 480 Perkin Elmer PCR machine

total amount run on 1% TBE agarose gel



Gel purification of 3.8kb bands:

approx wt. of gel fragments ~0.8g = 900 μ l

used QIAquick gel purification kit

eluted in 20 μ l H_2O + set up ligation

Ligation

7.5 μ l DNA1.0 μ l ligase1.5 μ l 10X ligation buffer5.0 μ l H_2O 15.0 μ l

inc. @ RT O/N

Continued on

Page #: 68

Book #: 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

P. Campbell

M. Chaka

M. Bernard

18/3/98

Campbell

18/03/98

M. Chaka

Signed

Dated

Signed

Dated

SEQUENCING: PCR BEV. 2 clones #4 & #7

PCR BEV. 3 clones #1 & #5

Date

18/3/98

Continued From

Page #: 64

Book #: 32

Aim: to sequence 2 clones of each to check BEV inserts.

Method: new primers arrived (x Derek Skingle)
x 4 for each primer

Big Dye	8.0ml	32.0
10µM primer	0.2	0.8
DNA (miniprep)	4.0	-
H ₂ O	7.8	31.2
	20.0	64.0

Seq. run		Primer	Label
1	PCR BEV. 2 clone #4	F	QMG 40
2	" "	R	41
3	" clone #7	F	42
4	" "	R	43
5	PCR BEV. 3 clone #1	F	44
6	" "	R	45
7	" clone #5	F	46
8	" "	R	47

18/3/98

Clean up: 2µl 3M Na acetate
50µl 100% EtOH (RT)

↓
-20°C / 12 hrs.

↓
14K / 30' @ 4°C

↓
1x 70% wash

↓
Speedi vac to dry pellet

NB. not all volumes
after sequencing run
looked equal.
preparing a master mix
may not be very good!

Continued on

Page #:

Book #:

Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Graham

1993

19/3/98

Campbell

19/03/98

Graham

24/3/98

Dated

Signed

Dated

Dated

TRANSFORMATIONS: pcmv.cass

Date

19/3/98

Continued From

Page # 66

Book # 32

Aim: to transform ligation from p.66 into DH5A

Method:

cells

Ampl. ligation

DH5A chemically competent

① 50µl

5µl

② 50µl

10µl

Heat shock protocol
Recovery in 400µl SOC

plated out 2x100 + 200µl onto LB+kan 50µg/ml

19/3/98

20/3/98

Transformation # colonies

1

19 + 12

2

22 + 15

Next: set up miniprep cultures (LB+kan 50µg/ml)

22/3/98

12 colonies selected + miniprep cultures
set up

2ml LB+kan 50µg/ml

inc. 37°C O/N.

Continued on

Page # 69

Book # 32

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Bernard

P. Campbell

M. C. C.

M. Bernard

22/3/98

P. Campbell

22/03/98

M. C. C.

Signed

Dated

Signed

Dated

Signed

23/3/98

Qiagen spin columns

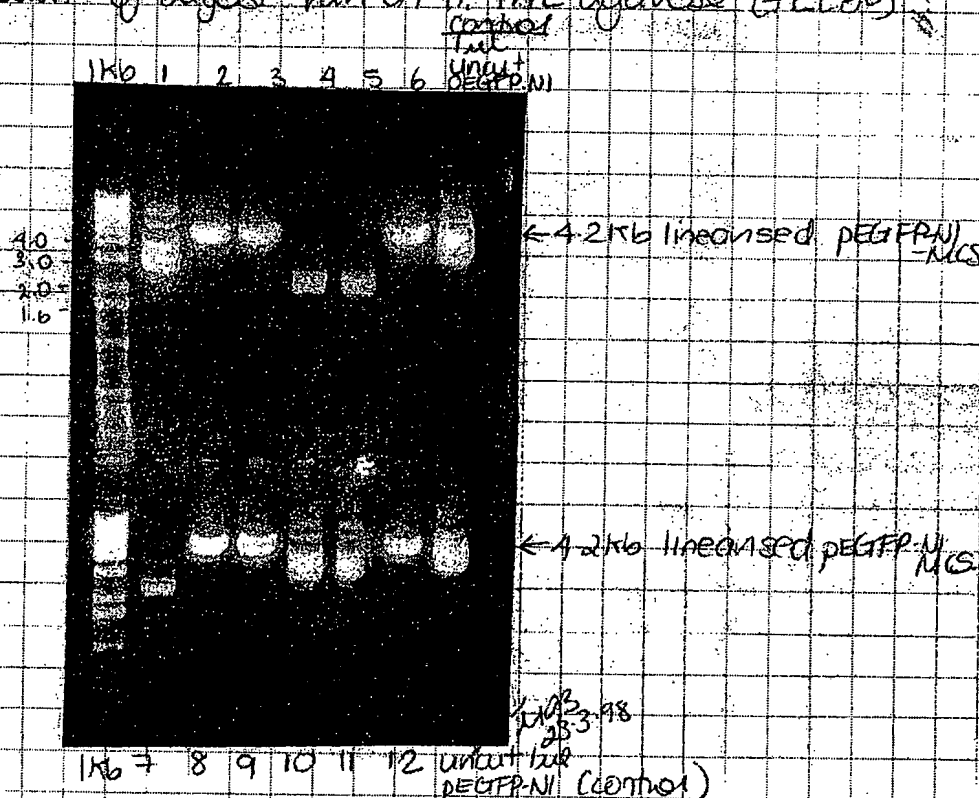
eluted in 100 μ l H₂O5 μ l DNA digested with NotI

clones (if correct) should have lost the NotI site
after end filling with Pfu & religating. \therefore correct clones
should run as uncut vector, but smaller (\therefore faster)

Digests:

DNA	5 μ l	x12	
10X buffer H	2.0	24.0	
100 μ M NaCl	0.5	6.0	
H ₂ O	12.5	150.0	
	20.0	180.0	inc 37°C / 2 hrs

Total amount of digest run on 1% TAE agarose (GEEB)



Comments: clones #4, 5 & 7 are running as uncut & faster
than control \rightarrow check further with other RE
- clone #1, 10 & 11 are running as uncut & only slightly
faster than control \rightarrow maybe check one of these clones

Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

Bernard

P. Campbell

M. Curran

Bernard 23/3/98

Campbell

23/03/98

Curran

23/3/98

Dated

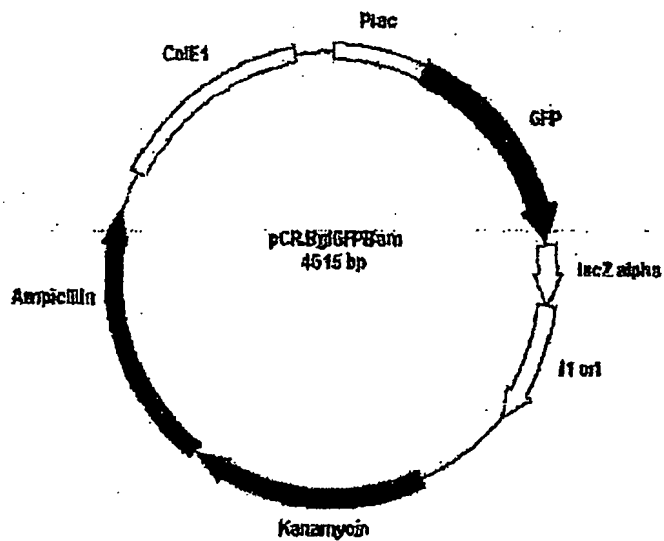
Signed

Dated

Signed

Dated

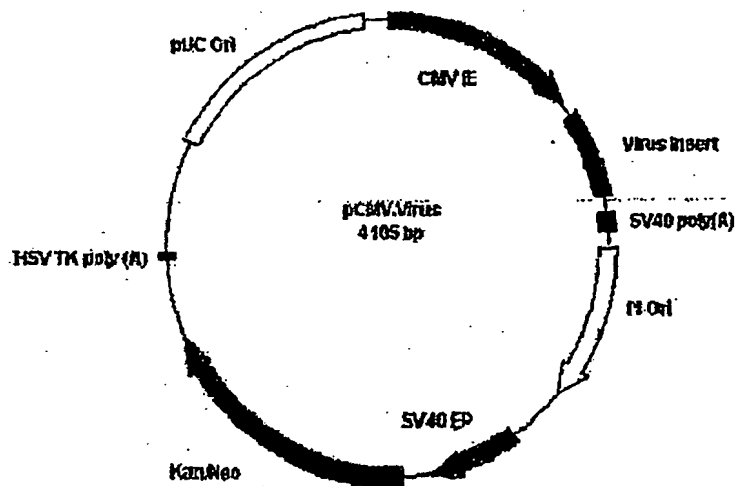
EXHIBIT 8



Author:
Date:
Notes:

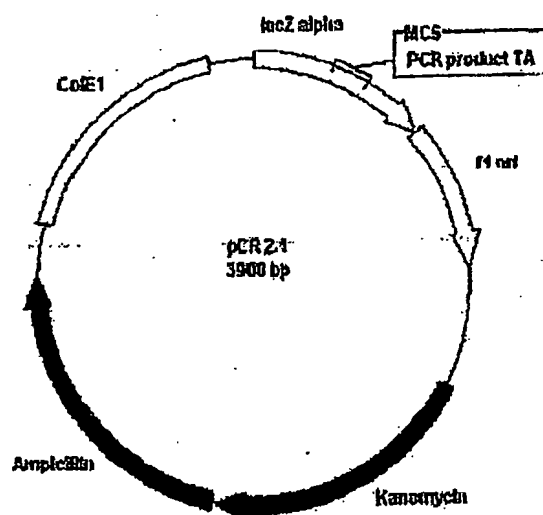
PBG40LBA-PLA

Created 21/01/1998



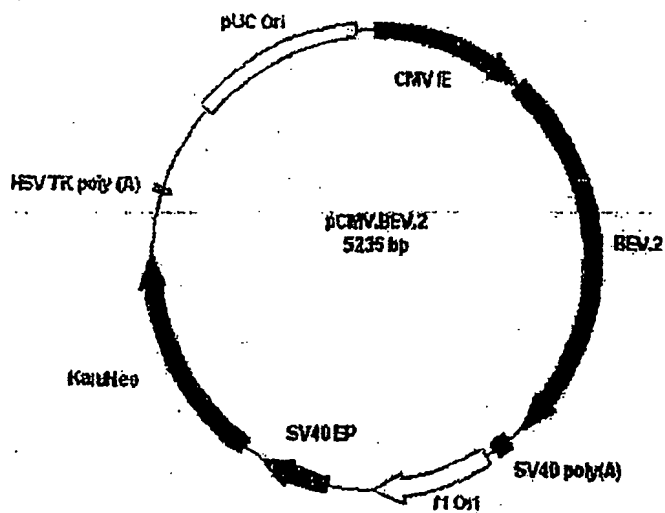
Author:
Date:
Notes:

PCMV-VIR. PLA
Created 21/01/1998



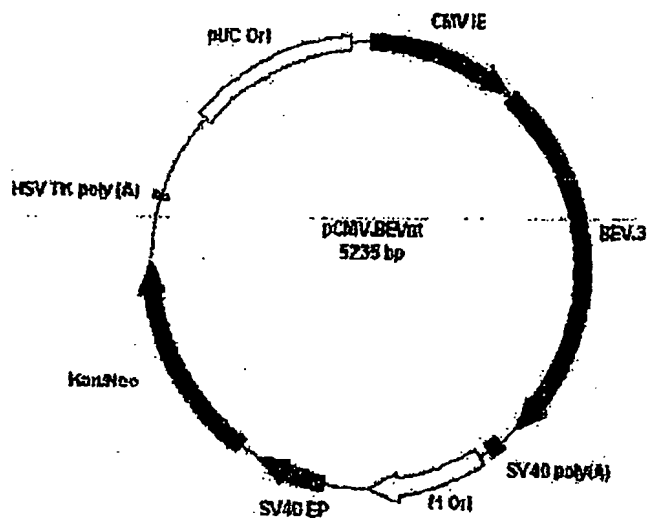
Author:
Date:
Notes:

pCR2.1.PLA
created 2/01/1998



Author:
Date:
Notes:

PCMV/BEV2:PLA
created 22/01/1992

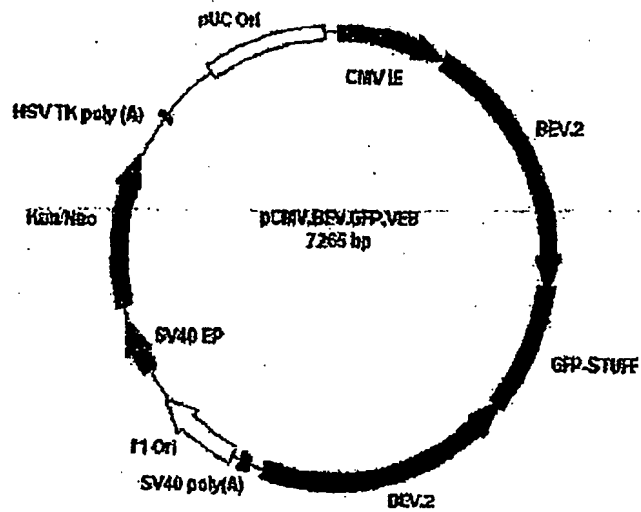


nt = Non-translatable

Author:
Date:
Notes:

PCMV.BEV3: PLA

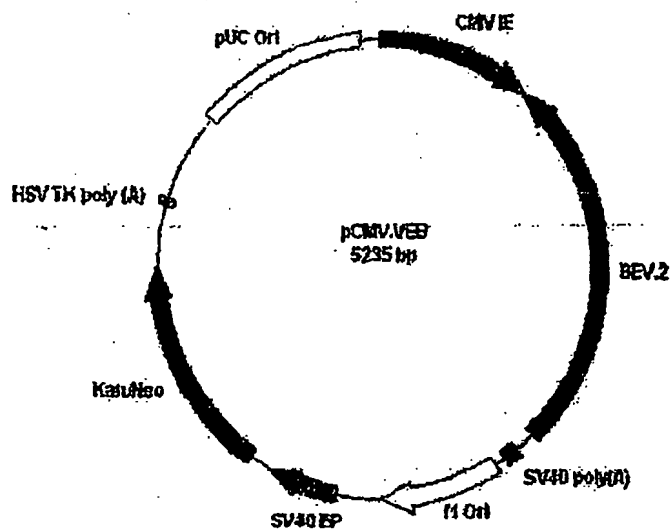
created 22/01/1998



Author:
Date:
Notes:

PCMV.BGV-PLA

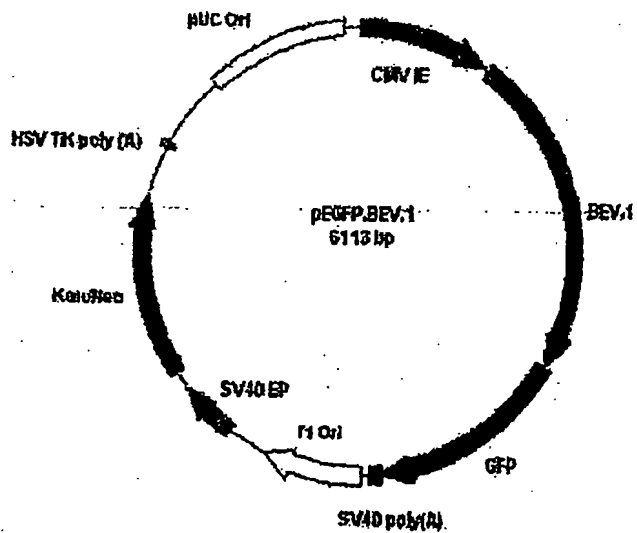
Created 22/01/1998



Author:
Date:
Notes:

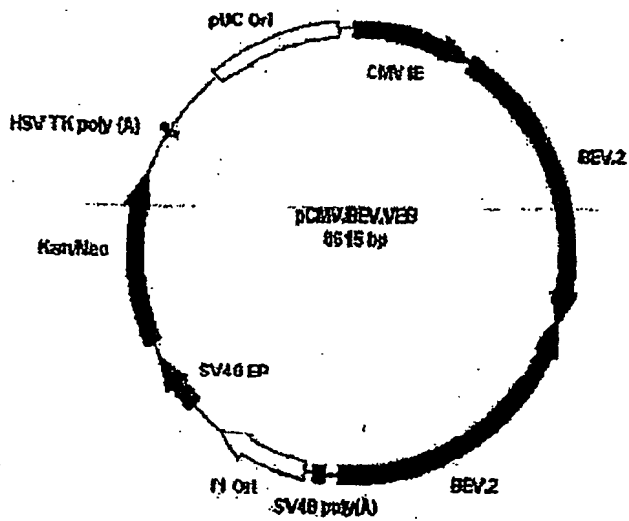
PCMV VEB2.PLA

Created 22/01/1998



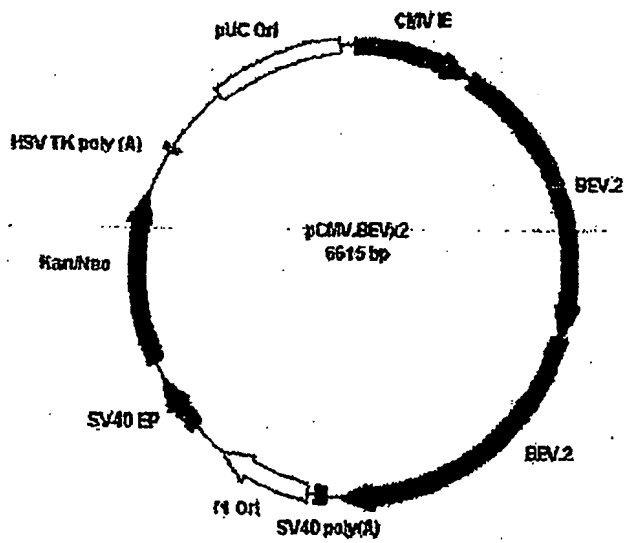
Author:
Date:
Notes:

PEGFP.BEV. PLA
Crabtree 22/01/1998



Author:
Date:
Notes:

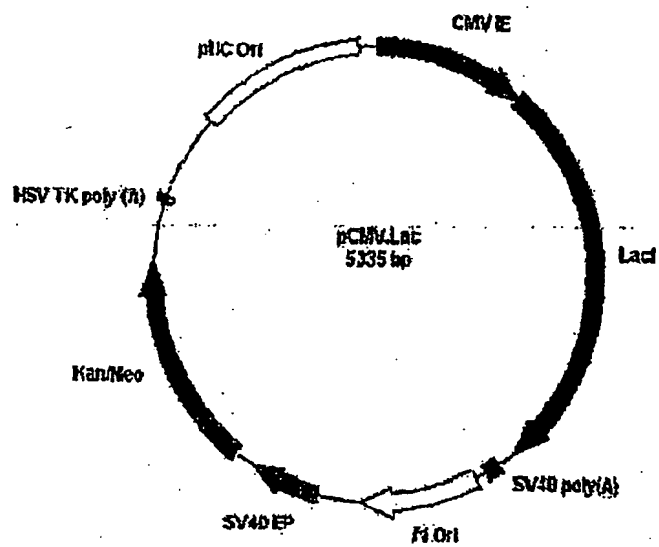
PCMV BEV.VES
Created 22/01/1998



Author:
Date:
Notes:

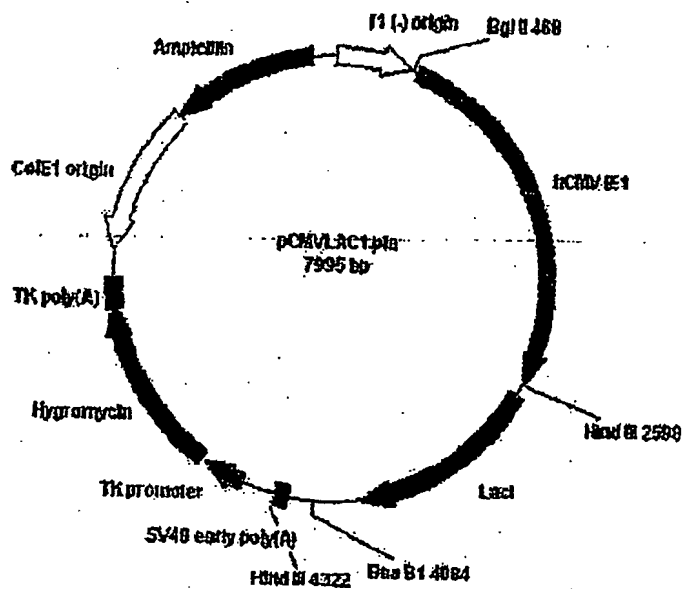
pCMV.BEV2.X2

Created 22/01/1998



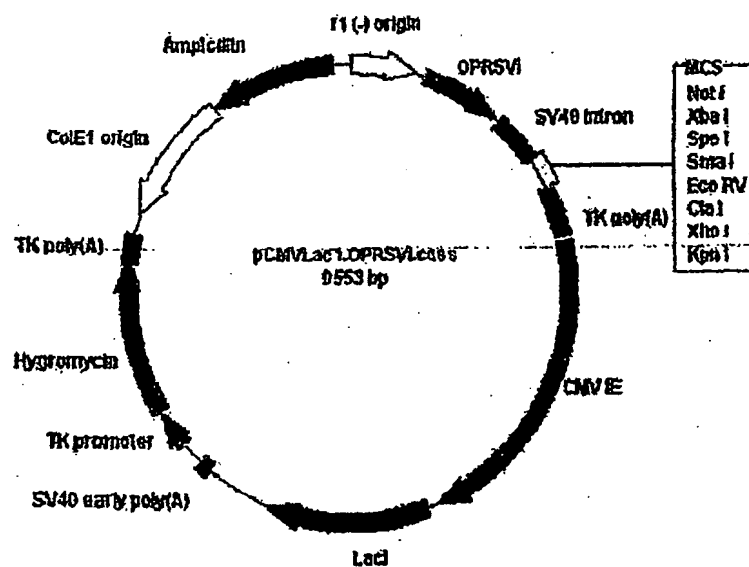
Author:
Date:
Notes:

PCMV-LAC-PLA
created 25/02/1998



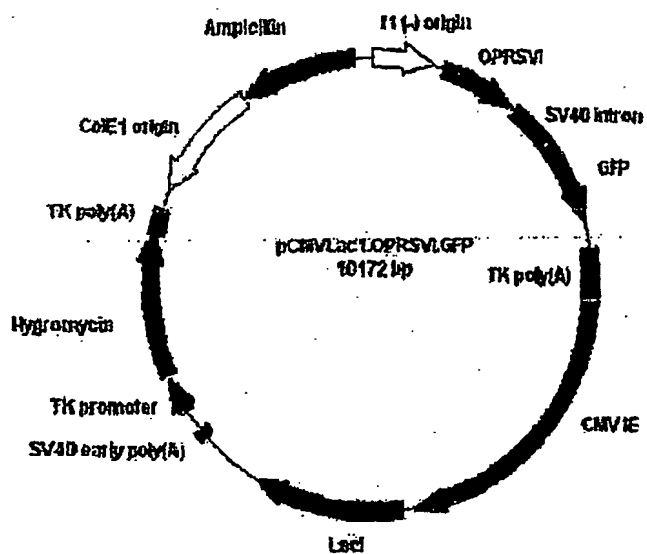
Author:
Date:
Notes:

PCMV LAC1. PIA
created 25/02/1998



Author:
Date:
Notes:

PLMVORLS.CAS
Created 26/02/1998



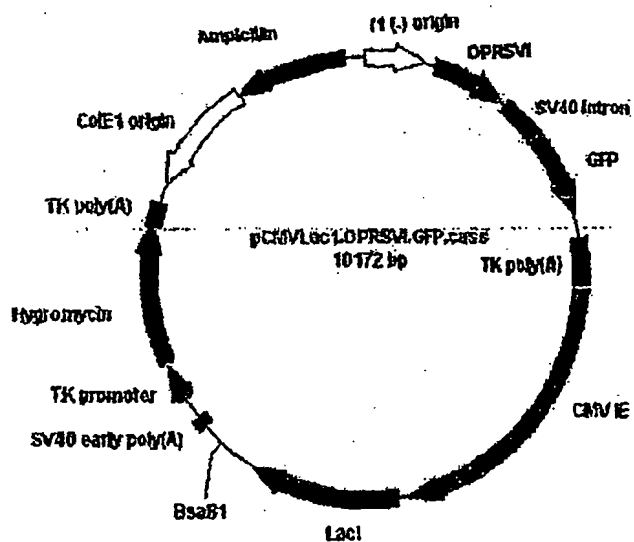
Author:

Date:

Notes:

CMOPRGFP. PLA

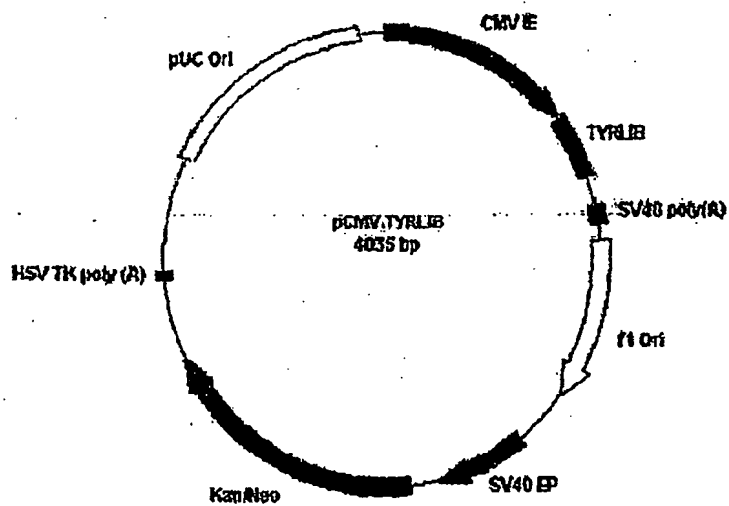
Created 26/02/1998



Author:
Date:
Notes:

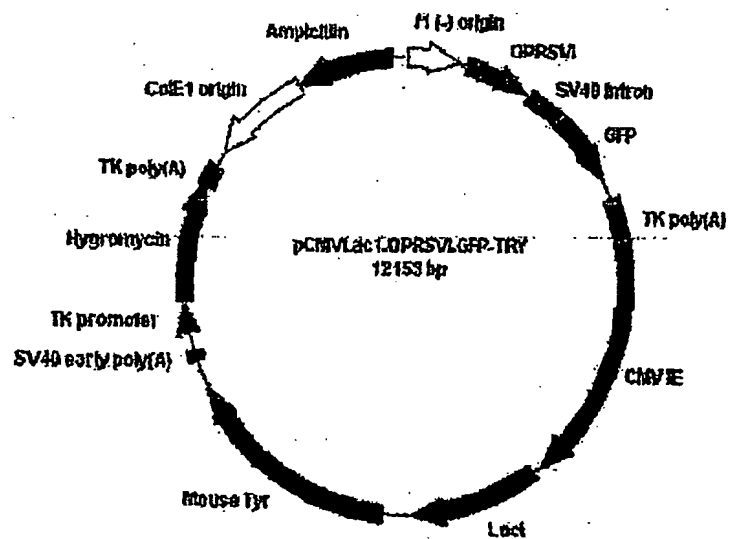
CMOPRGFP.CAS

created 27/02/1998



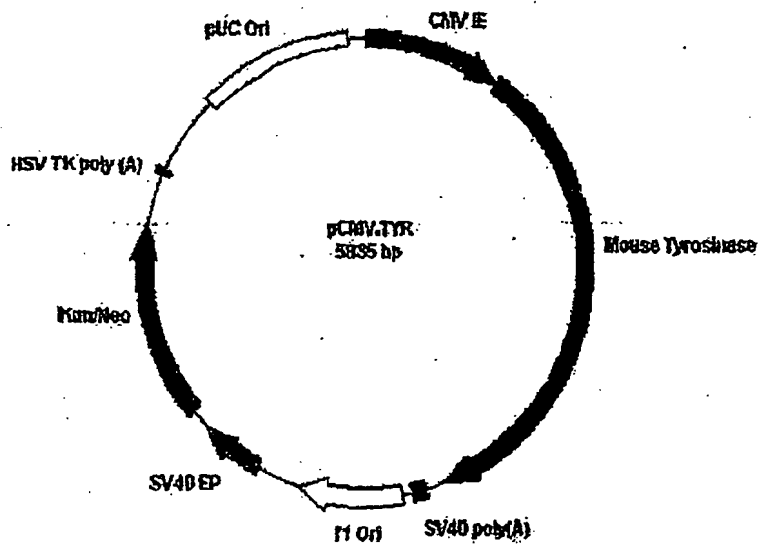
Author:
Date:
Notes:

CMV.TYRLI. PLA
Created 27/02/1998



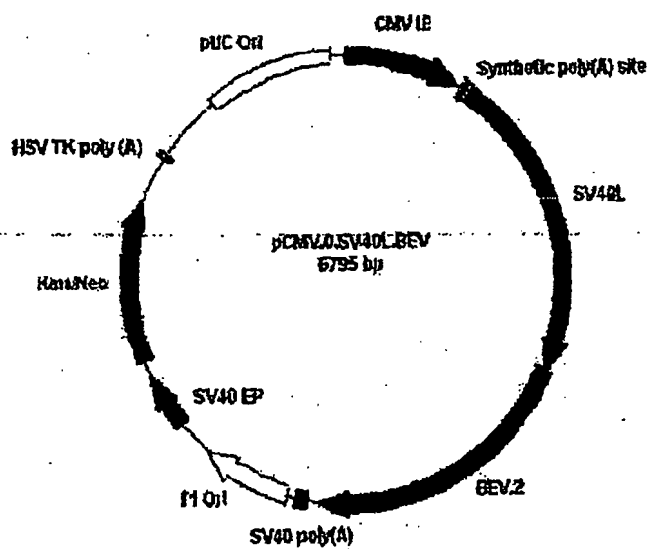
Author:
Date:
Notes:

CMOPRGFT. PLA
Created 27/01/1998



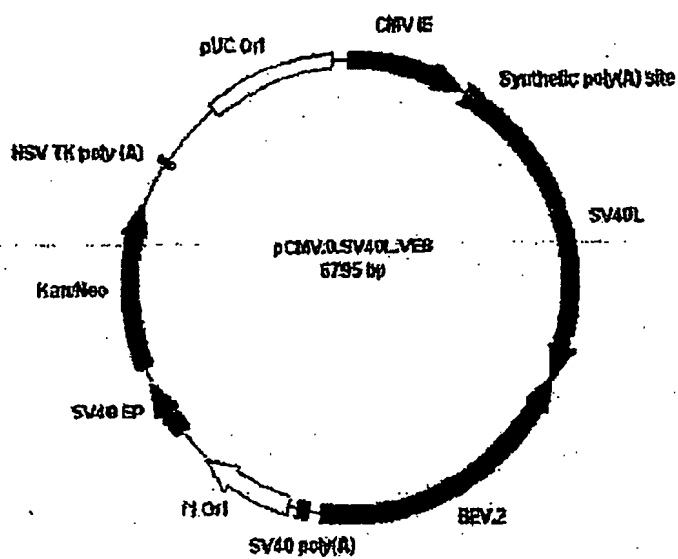
Author:
Date:
Notes:

CMVTRY.PLA
Created 2/03/1998



Author:
Date:
Notes:

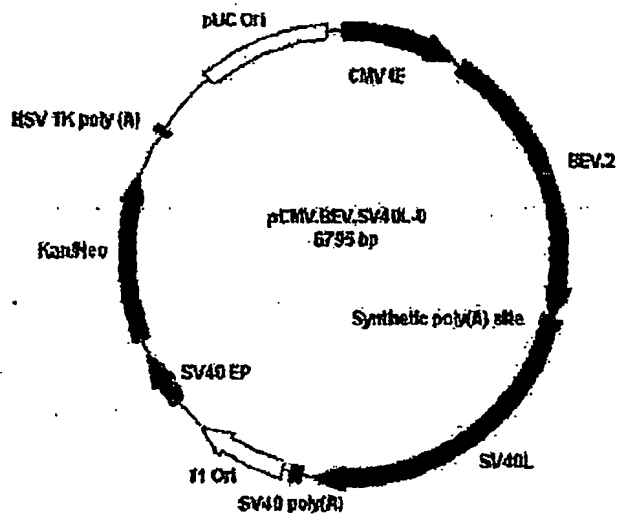
File 03V40BE.pla
Created 5/03/1998



Author:
Date:
Notes:

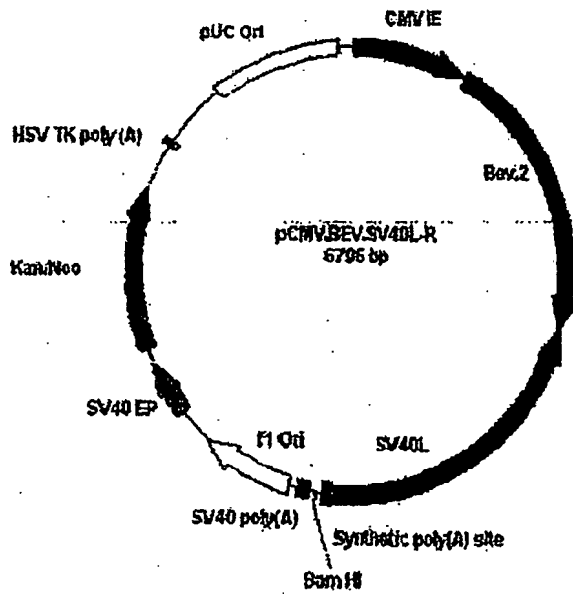
OSV40VEB. Pl₂

Created 5/03/1999



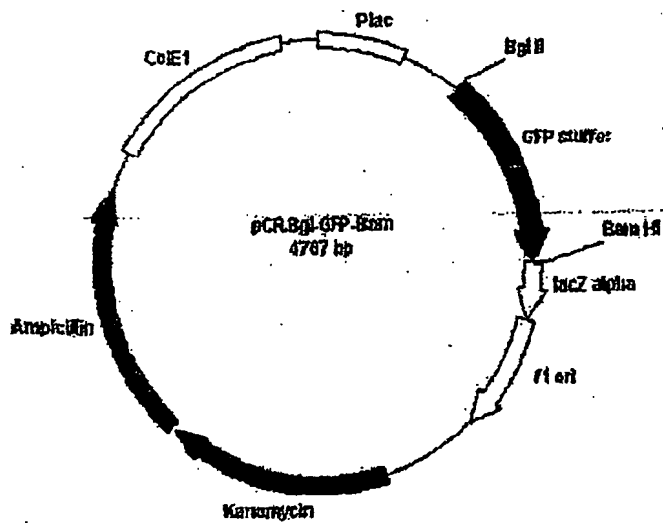
Author:
Date:
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BE_S40_0.1/a
Created 5/03/1998



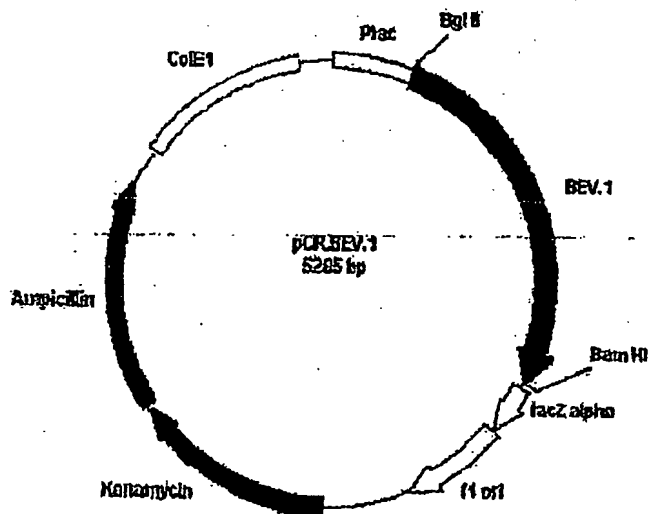
Author:
Date:
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Created 5/03/1998



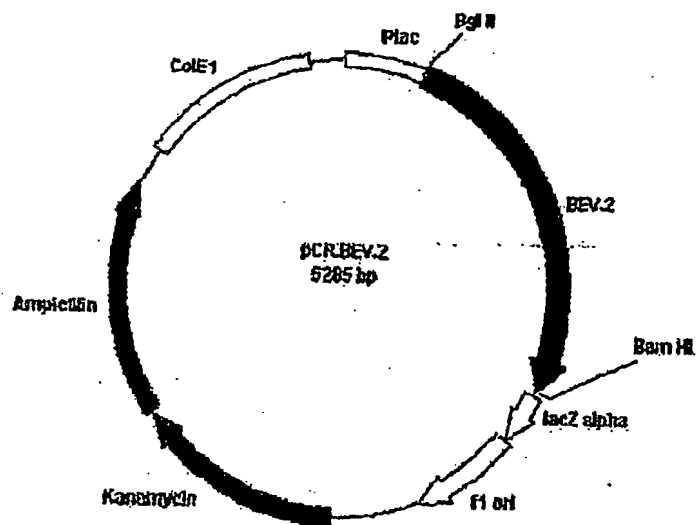
Author:
Date:
Notes:

PCB-GFP-BA. PLA
created 5/03/1998



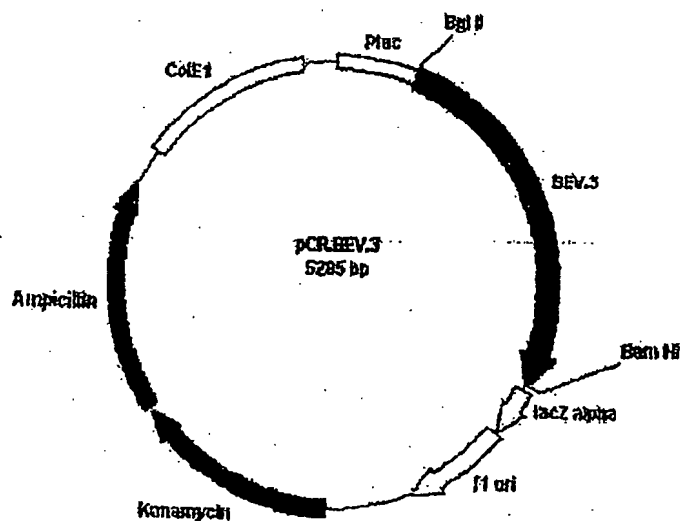
Author:
Date:
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PCR-BEV.1. PLA
created 5/03/1998



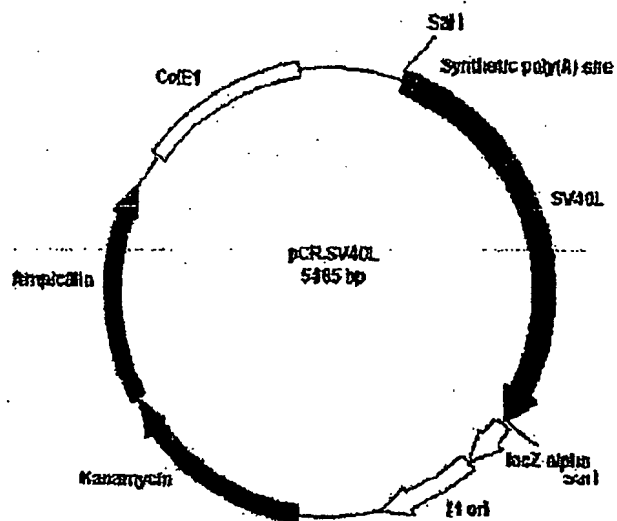
Author:
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Notes:

PLA-BEV.2-PLA
Created 5/03/1998



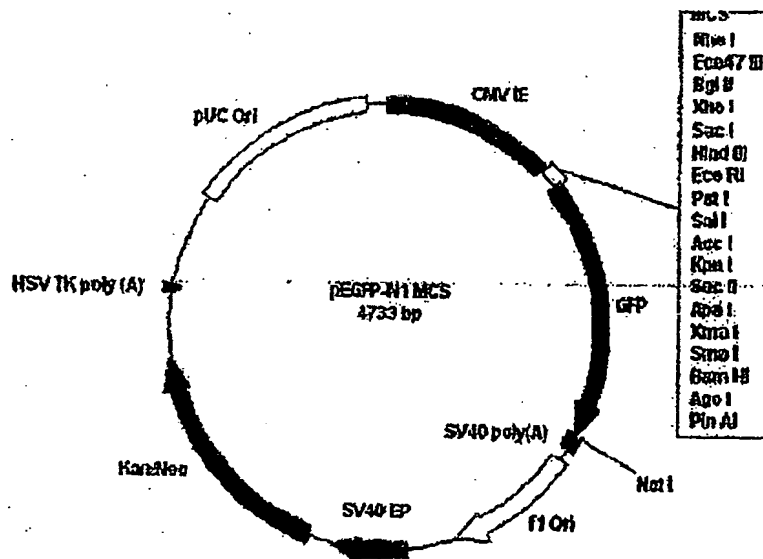
Author:
Date:
Notes:

PCR-BEV3.PLA
created 5/03/1998



Author:
Date:
Notes:

pCR.SV40L. PLA
created 5/03/1998



Author: Robert Rice

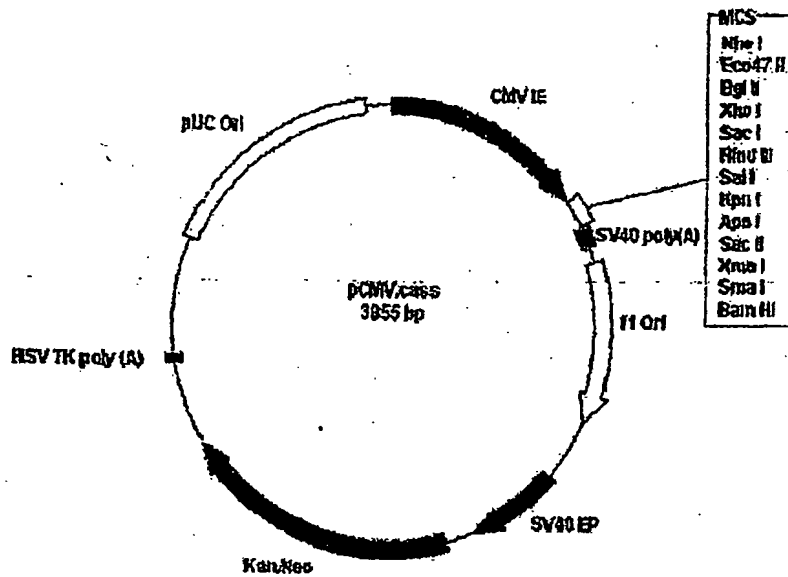
Date: 22/1/98

Notes:

Expression cassette: pEGFP-N1MCS: A
commercially obtained vector (CLONTECH)
from which most expression constructs are
be derived.
to

PEGFP-N1 PLA

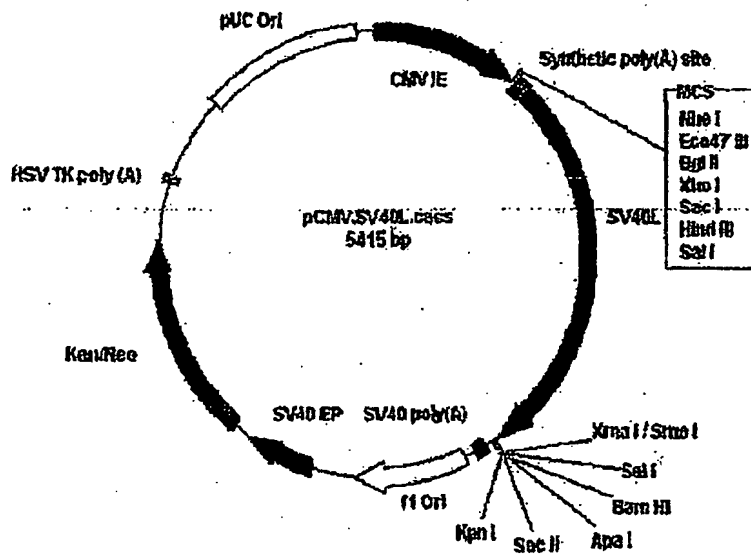
Created 5/03/1998



Author:
Date:
Notes:

PCMV.CAS

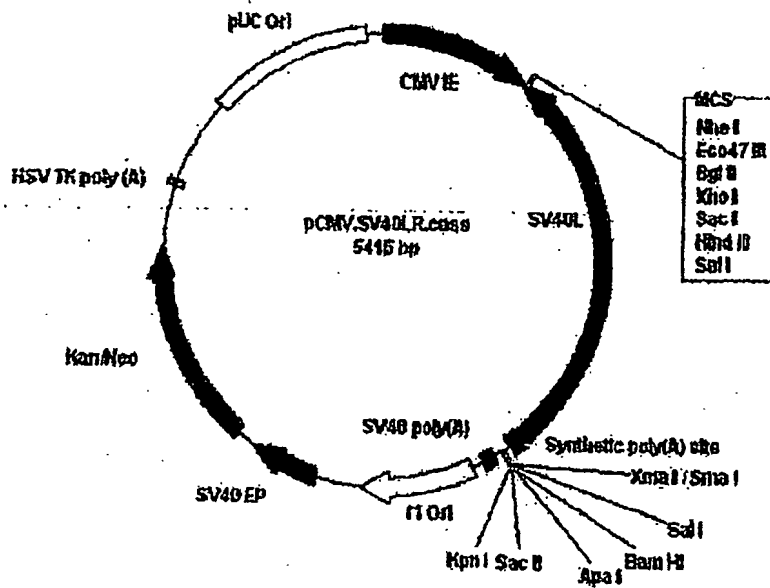
created 6/03/1998



Author:
Date:
Notes:

PCMV SV40.CAS

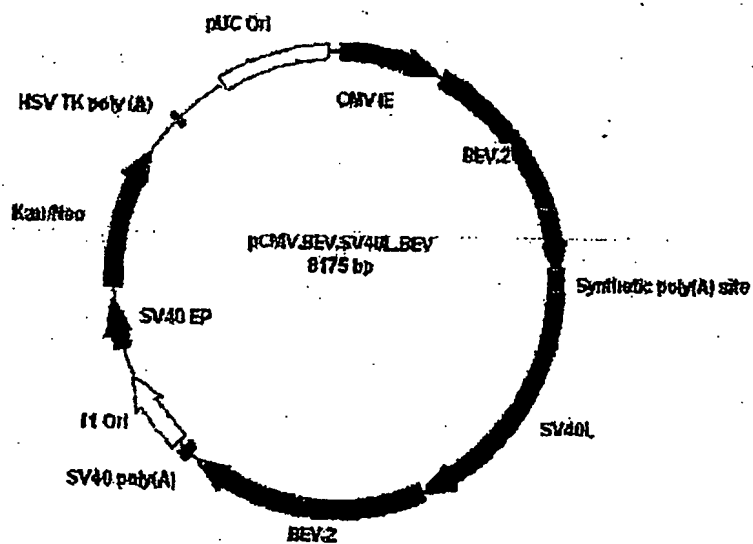
Created 6/03/1998



Author:
Date:
Notes:

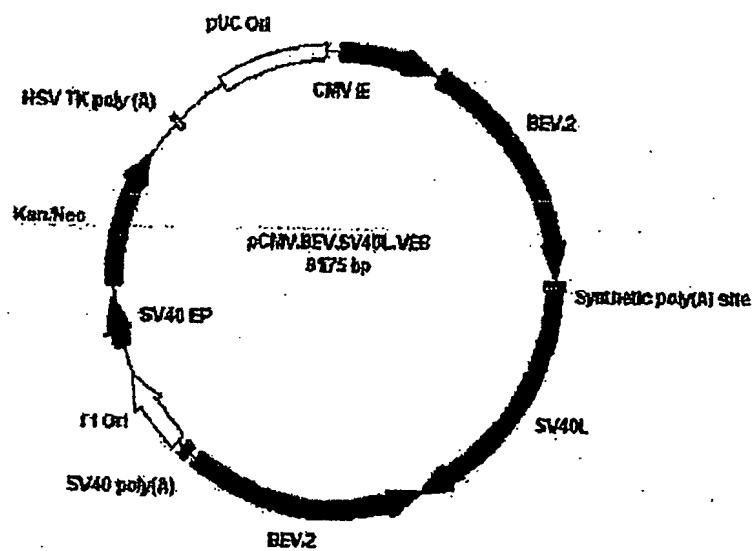
PCMV SV40.L.CAS

Created 6/03/1998



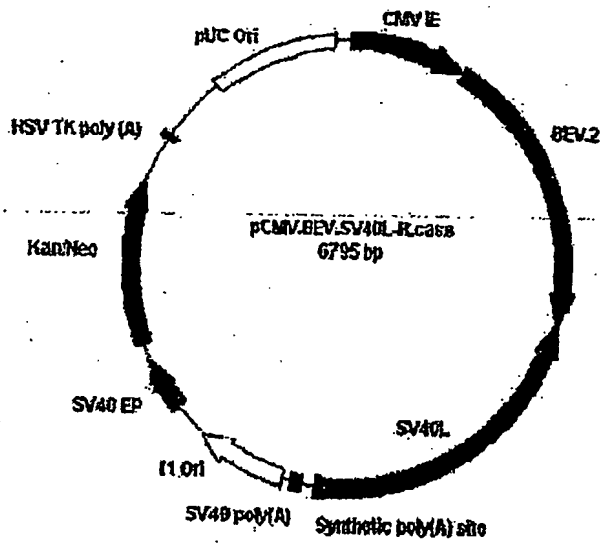
Author:
Date:
Notes:

BEV SV BEV. P/A
created 6/03/1998



Author:
Date:
Notes:

BEVSVVES.P/A
Created 6/03/1998



Author:
Date:
Notes:

CMBE SV4R. PIA
Created 6/03/1998

EXHIBIT 9

Transferring MDRK cells with pEGFP.BEV.1

Page

1

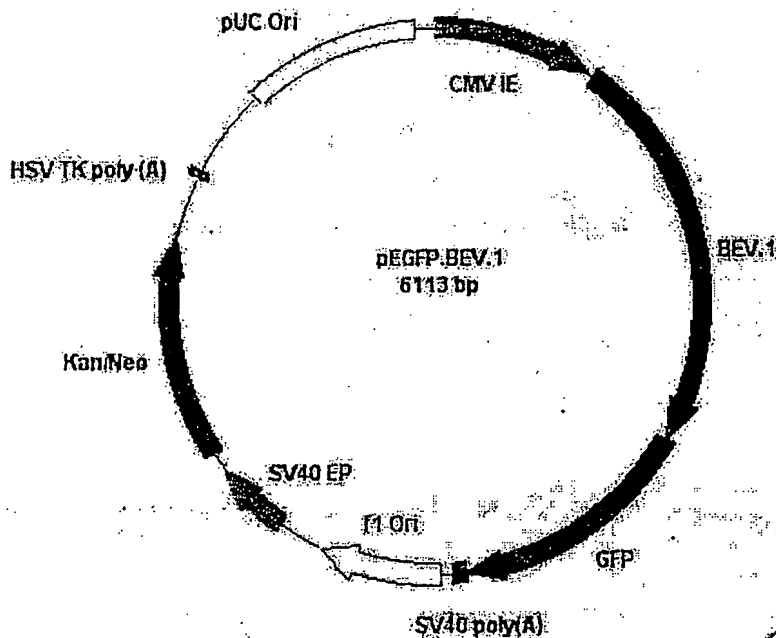
Date

11/3/98

Continued From

Page #

Book #



Encodes
BEV.1
GFP

fusion rate

Have split MDRK cells on Monday 11/3/98
For passage to week 2

Set up transfection on Wednesday

Prepared lipofectamine 2 μ l DNA (~1 μ g)
100 μ l OPTI MEM

For each well

100 μ l (10 μ l lipofectamine
+ 90 μ l OPTI MEM)

18 wells
wells
1,2
3,4
5,6
7-12
13-18

Treatment
no DNA
no DNA
GFP control (pEGFP-N1)
pEGFP.BEV.1
pEGFP.BEV.1

Selective
none
Select 400 μ l sample

Continued on

Page #

41

Book #

41

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. C. Graham

K. Reed

John M. Kelly

11/3/98

Dated

Signed

11/3/98

Dated

11/3/98

Signed

Dated

Date

Continued From

Page #

Book #

Mixes prepared

1-4 50ml Lipofectamine + 4ml OPTIMEM

5-6 30ml Lipofectamine + 2 ml OPTIMEM
50ml MEM OPT
4ml PECP-DMES (1.2ml)

7-12 70ml Lipofectamine + 5.6 ml
1.30ml OPTIMEM
12-18 7ml PECP-DMES
RED-GFP

Left mixes ~1.5 hrs before adding 2nd aliquot
of media

Cells washed 2x 1ml OPTIMEM (1-12)
2x 0.5ml (13-18)

Added 1ml DMEM mix

Leave 2 hrs. then add media

50ml MEM	20
5ml FCS	10
5ml non-essential a.a.	2
5ml antibiotics	2
5ml Na Bicarbonate	6

Continued on

Page #

Book #

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Gurevich

KE REED

PIANO MCCREARY

Signed

Dated

Signed

Dated

Signed

Dated

11/3/98

K. Reed

11/3/98

Piano McCrery

11/3/98

Expt 1

Page

3

Date

17/3/98

Continued From

Page #

Book #

12/3/98 Changed medium OPTI media replaced with 1ml MEM.
13/3/98 Changed media for selection.
(Control 320ml / 40ml MEM)

1-2 normal MEM
3-18 MEM + gentamicin

GFP status

5+6 heaps of GFP the cells
(~10/field)
both live

7-18 GFP the cells apparent - very few ($\leq 10 \times$ less) than control

Fusion protein not as active as native protein or something else (Mitt stat 20.2)

Will change media every 2 days + monitor selection

16/3/98

Cultured flasks

Washed 2x with PBS

Treated with trypsin - 10min

Cultured on 2 flasks 200k cells \rightarrow 40ml MEM

For experiments cultured 6x6 well plates
0.36ml cells + 180ml media

17/3/98 Changed media for expt 1
Selection looks fairly good.

Continued on

Page #

Book #

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Givah

T. O'Hare

P. Campbell

[Signature]

17/3/98

[Signature]

20/3/98

[Signature]

20/03/98

Dated

Signed

Dated

Signed

Dated

Expt 2: Kill curve for granulosa cells
 Expt 3: Direct selection for BEU immaturity

2. Transformation

Expt 2: 3 plates - PCFP - U1 MCS for select
 Expt 3: " PCFP, BEU CFP

Prepare DMEM in 20 TIF

	Expt 2	Expt 3
DMEM	200ul	200ul
Lipid solution	1.2ul	1.25ul
OPT MEM		
45 in AT		
100	100ul OPT MEM	100ul OPT MEM
		Shuffled up and mixed in 10 TIF by pipetting
		Make CFP

Wash cells 2x in OPT MEM

1ml of above mixed to plate

Expt 2	Kill curve	Set 50mg/ml
	refuse to grow	refuse to grow
1, 2	200	200
3, 4	300	300
5, 6	400	400
7, 8	500	500
9, 10	600	600
11, 12	700	700
13, 14	800	800
15, 16	900	900
17, 18	1,000	1,000

20/3/88

Change media for Expt 1

2

3

Expt 11,2 change media to gentamicin
2-K " " + 400 ug/ml gentamicin3 One media + ~~250 ug~~ gentamicin
500 ugExpt 2

Kill curve, as on previous page

Expt 3Change media, will infect cells
Monday with REU

Name of Person Conducting Experiment

First Witness of Experiment

Second Witness of Experiment

M. Graham

P. Campbell

Tim O'Brien

Signed

Dated

Signed

Dated

Signed

Dated

20/3/88

20/03/88

20/3/88